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13. ABSTRACT ***(Maximum 200 Words)***

This report describes progress made during the first year of support for a translational research project involving *Nf1* mutant mice with myeloid leukemia. This study has two Technical Objectives. First, examining the therapeutic efficacy of two agents (1) mycophenolate mofetiel (MM) and, (2) a fusion toxin that targets the GM-CSF receptor. These compounds represent rational new approaches for treating NF1-associated tumors. MM has been tested in the mouse model and our preliminary data indicate that it is unlikely to provide benefit to NF1 patients. We have also produced and purified the GM-CSF immunotoxin for use in preclinical studies during the next fund year. We are also performing correlative biochemical studies to elucidate the effects of these therapeutics on cellular GTP levels and Ras signaling. In aim 2, we are utilizing *Nf1* mice to extend clinical observations suggesting that individuals with NF1 are susceptible to the development of therapy-associated second cancers. These studies were initiated over the past year. We will perform other correlative molecular studies in mice that develop tumors. We anticipate that the proposed experiments will yield novel data that may be of practical value to patients with NF1 and their physicians.

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(5) INTRODUCTION

Individuals with neurofibromatosis type 1 (NF1) are predisposed to specific benign and malignant neoplasms including juvenile myelomonocytic leukemia (JMML; formerly known as JCML). Clinical data also suggest that children with NF1 are at increased risk of developing leukemia as a complication of genotoxic therapies for another primary cancer. Genetic and biochemical studies of leukemia samples from children with NF1 performed in our laboratories have shown that *NF1* functions as a tumor suppressor gene in immature hematopoietic cells by negatively regulating the Ras signaling pathway (Bollag et al., 1996; Kalra et al., 1994; Miles et al., 1996; Shannon et al., 1994; Side et al., 1997). Similarly, heterozygous *Nf1* mutant *(Nf1+/-)* mice show an increased incidence of myeloid leukemia and other cancers (Jacks et al., 1994). We recently reported that treatment with the alkylating agent cyclophosphamide cooperates strongly with heterozygous inactivation of *Nf1* in murine leukemogenesis (Mahgoub et al., 1999). Homozygous *Nf1* mutant embryos *(Nf1-/-)* die *in utero.* Like human JMML cells, *Nf1-/-* fetal hematopoietic cells display a selective pattern of hypersensitivity to the cytokine growth factor GM-CSF in myeloid progenitor colony assays (Bollag et al., 1996; Largaespada et al., 1996). Adoptive transfer of these cells consistently induces a myeloproliferative disorder (MPD) that resembles JMML in irradiated recipients (Largaespada et al., 1996; Zhang, 1998). The predictable nature of this syndrome, the fact that transplanted mice survive for many months, and the well-characterized biochemical alterations in *Nf1*-deficient hematopoietic cells make this model attractive for testing novel therapeutics and for biologic studies of growth control.

The approved Statement of Work for this translational research project has two Technical Objectives which we are pursuing through two Specific Aims. Aim 1 proposes preclinical studies in recipient mice that have been transplanted with *Nf1*-deficient fetal liver cells to investigate the therapeutic efficacy of two agents (1) an inhibitor of *de novo* guanine nucleotide synthesis and, (2) a recombinant fusion toxin that targets the GM-CSF receptor. These compounds were chosen because they represent rational new approaches for treating NF1-associated tumors. We are also performing correlative biochemical studies to elucidate the effects of these therapeutics on cellular GTP levels and Ras signaling. In aim 2, we are utilizing *Nf1* mice to extend clinical observations suggesting that individuals with inactivation of one *NF1* allele are susceptible to the development of therapy-associated second cancers. We are exposing cohorts of wild type and *Nf1+/-* mice to either radiation therapy alone, or to radiation combined with cyclophosphamide to test the hypothesis that these mutagens will cooperate with each other and with inactivation of *Nf1* in tumorigenesis. We will examine tumor tissues for loss of heterozygosity (LOH) at *Nf1* and will perform other correlative molecular studies. We anticipate that the proposed experiments will yield novel data that may be of practical value to patients with NF1 and their physicians.

(6) BODY

Technical Objective (Aim) 1: Testing Rational Therapeutics in Nf1 Mice

Overview of Preclinical Therapeutic Studies. This component involves independently testing the efficacy of two rational therapeutics to inhibit the growth of *Nf1-/-* hematopoietic cells *in vivo,* and performing correlative biochemical and cell biologic assays. Our progress is described below.

Preclinical Evaluation of Mycophenolate Mofetiel (MM). As described in our proposal, MM is an inhibitor of the enzymoe inosine 5' monophosphate dehydrogenase (IMPDH), which is required for *de novo* guanine nucleotide synthesis. The rationale for investigating this agent is based upon the idea that therapeutics that lower the ratio of GTP to GDP in the cell should decrease Ras-dependent growth because the activation state of Ras depends on the selective binding of GTP. The murine transplant model is ideal for initial studies that examine the efficacy of GTP reduction in inhibiting the abnormal growth of *Nf1*-deficient cells because the biochemical consequences of gene inactivation are well characterized in hematopoietic cells and because these cells rely heavily on the *de novo* pathway for nucleotide biosynthesis. Indeed, MM has shown anti-tumor efficacy in a number of preclinical studies performed in athymic nude mice. Additional background information, including calculations which suggested that cells in which an oncogenic *RAS* mutation or loss of *Nf1* would show enhanced sensitivity to a reduction in intracellular GTP concentrations, are presented in our proposal.

In year 1, the technical objectives related to the MM component of this project were (1) to treat normal mice at three dose levels of MM, to monitor clinical responses, and to examine diseased tissues at necroscopy; (2) to breed mice and perform adoptive transfer experiments to generate recipients repopulated with wild type or *Nf1-/-* fetal liver cells; (3) to select a dose of MM for preclinical evaluation; and, (4) to undertake correlative biochemical studies.

MM has been approved by the FDA for use in organ transplant rejection under the trade name Cell Cept. Based on preclinical data showing that MM is well-tolerated in mice at doses up to 240 mg/kg/day with diarrhea and anemia as the dose-limiting toxicities, we first treated cohorts of wild type F1 mice from a cross between strains C57BL6 and 129Sv with either 100, 150, or 200 mg/kg/day of MM. The drug was given as a single daily oral gavage dose for 8 weeks. MM was well-tolerated at these doses and, as shown in Figure 1, we observed no significant changes in blood cell counts. Given the lack of hematologic or other significant toxicities in the initial cohorts of C57BL6/129Sv F1 mice, we administered 300 or 400 mg/kg/day of MM to additional animals for up to 20 weeks. These doses were generally well tolerated, although mice treated at both doses showed weight loss and anemia (Figure 2A). White blood cell counts and myeloid counts declined after 6 weeks, but rebounded and stabilized thereafter (Figure 2B).

Based on these pilot data in wild type mice, we initiated a preclinical trial in which irradiated recipients engrafted with either *Nf1+/+* or *Nf1-/-* fetal liver cells were randomly assigned to receive either MM at a dose of 400 mg/kg/day, or a vehicle control, for 8 weeks. These animals were entered at least 12 weeks after adoptive transfer. All of the recipients of *Nf1-/-* fetal liver cells had leukocytosis with elevated myeloid cell counts at entry, and engraftment with *Nf1*-deficient cells was verified by Southern blot analysis. We evaluated 4 groups (1) mice reconstituted with *Nf1+/+* cells that received MM; (2) mice reconstituted with *Nf1-/-* cells that received MM; (3) untreated mice reconstituted with *Nf1+/+* cells; and, (4) untreated mice reconstituted with *Nf1-/-* cells. The results of this experiment are shown in Figures 3 and 4. Surprisingly, treatment with MM was associated with a dramatic rise in total leukocyte and myeloid cell counts in recipients engrafted with *Nf1-/-* or wild type fetal liver cells. White blood cell counts in excess of 100,000 per mm^3 were detected in some *Nf1-/-* recipients, and bone marrow smears revealed myeloid hyperplasia. The lack of statistical significance in MM-treated mice engrafted with *Nf1-/-* fetal liver cells after 8 weeks is due to the fact that 2 animals that had white blood cell counts > 100,000 per mm^3 after 6 weeks died before the end of treatment. Spleen weights were greater in wild type recipients that received MM than in untreated controls

(mean spleen weight 0.21 gm vs 0.03 gm in the untreated mice; p = 0.004), but not in *Nf1-/-* recipients (0.39 gm vs. 0.35 gm in untreated mice with MPD). In contrast, red blood cell counts declined in both cohorts of MM-treated mice, although this difference did not achieve statistical significance in the *Nf1-/-* group (Figure 4). We are presently analyzing liver and spleen sections from these mice. It is not clear why we observed a dramatic leukocytosis in MM-treated transplant recipients, but not in the wild type mice treated in our pilot experiments. We are presently treating additional untransplanted wild type mice in an effort to address this question.

Drs. Bollag and Shannon have initiated cell biologic and biochemical studies to further elucidate the basis of this unexpected response to MM. Assays of bone marrow colony forming unit granulocyte macrophage (CFU-GM) numbers revealed a decline in MM-treated *Nf1-/-* mice that did not achieve statistical significance, and a slight increase in the wild type controls (Figure 5A). In contrast, *Nf1-/-* recipients had a slight rise in splenic CFU-GM numbers from abnormally high baseline levels, while wild type mice showed a highly significant increase (Figure 5B). Thus, accelerated myelopoiesis in MM-treated wild type mice is associated with the appearance of substantial numbers of splenic CFU-GM. Our preliminary biochemical data indicate that exposing myeloid cells to mycophenolic acid (the active metabolite of MM) *in vitro* is associated with a reduction in cellular GTP levels.

Plan for the Next Year of Funding. The paradoxical increase in leukocyte counts seen in mice treated with MM preclude the therapeutic use of this agent in JMML. However, it is important to determine if we have succeeded in reducing GTP levels in primary cells and, if this is true, how this has altered Ras activation in resting and in growth factor-stimulated cells. We have previously measured mitogen activated protein (MAP) kinase signaling in primary cells from mice treated with a farnesyltransferase inhibitor (Mahgoub et al., 1999), and have developed assays to examine activation of Akt and Ras•GTP levels. One potential explanation is that reducing cellular GTP levels has had a pronounced effect on inhibitory GTP-binding proteins, and that this has led to unrestrained Ras signaling. Because our results have implications for the general strategy of reducing GTP levels in *Nf1*-deficient cells, we will examine the biochemical effects of MM on Ras signaling in myeloid cells over the next few months.

Preclinical Evaluation if a DT_{ct}GM-CSF Recombinant Fusion Toxin. Investigations of myeloid progenitor colony growth in JMML patients and *Nf1-/-* mice have implicated hypersensitivity to GM-CSF in leukemogenesis. Studies performed in our laboratory through NIH grant RO1 CA72614 since this Army proposal was funded provide additional support for this hypothesis. We developed embryos with homozygous disruptions of both *Nf1* and *Gmcsf* genes by breeding *Nf1* and *Gmcsf* mutant mice, and transferred these cells into irradiated wild type or *Gmcsf*-deficient recipients. Development of the JMML-like MPD that follows adoptive transfer of *Nf1-/-* cells was delayed, but not eliminated, in *Gmcsf-/-* hosts that survived with a graft of *Gmcsf-/- x Nf1-/-* cells. We also performed secondary adoptive transfer experiments in which bone marrow cells from wild type recipients that had developed MPD after receiving *Gmcsf-/- x Nf1-/-* fetal liver cell grafts was transferred into wild type or *Gmcsf-/-* hosts. Although all of the wild type recipients of these cells developed MPD, the *Gmcsf-/-* hosts did not. Furthermore, GM-CSF treatment promptly induced MPD in secondary recipients of *Gmcsf-/- x Nf1-/-* cells, but had no effect in mice reconstituted with *Gmcsf-/- x Nf1+/+* cells. Together these data, which are summarized in Figure 5, implicate GM-CSF as playing a central role in the pathogenesis of JMML and suggest that inhibiting this signaling pathway might provide therapeutic benefit.

One aspect of Technical Objective 1 involves production of highly purified recombinant DT_{ct}GM-CSF fusion toxin and *in vitro* and *in vivo* murine studies in the *Nfl* mouse model system with the goal of specifically delivering the diphtheria toxin to myeloid cells via the GM-CSF receptor. Substantial progress has been achieved in accomplishing this objective. The work in the first year of support has focused upon the large-scale production and purification of the murine DT_{ct}GM-CSF fusion toxin in at a high quality clinical grade appropriate for *in vivo* murine studies. We have successfully developed a refined expression and purification method for DT_{ct}GM-CSF that overcomes problems with product aggregation and denaturation during production when standard purification methods are employed. We have also initiated *in vitro* biochemical and functional characterization of DT-mGMCSF. Work is proceeding on-schedule for the investigations proposed for this research project.

DT_{ct}GM-CSF Immunotoxin Production. The structure of the recombinant murine growth factor-toxin fusion expression vector pET11d-DT-mGMCSF is shown in comparison to native diphtheria toxin in Figure 7A, and the construction of DT_{ct}GM-CSF is depicted in Figure 7B. In summary, a synthetic cDNA encoding murine GM-CSF using *E. coli* codon preferences was obtained from R & D Systems (Minneapolis, MN). The polymerase chain reaction (PCR) was used for mutagenesis of the murine *Gmcsf* gene to add NcoI and BamHI restriction enzyme to 5' end and 3' end respectively. The PCR primers for murine *Gmcsf* included the 5' primer mGM1F (5'-CCCATGGCACCCACCCGCTCACCC-3') and the 3' primer mGM1R (5'-GGGGATCCTCATTTTTGGACTGG-3'). The cloning vector pET11:mGMCSF was constructed by the cloning of the murine *Gmcsf* gene cassette downstream of the T7 promoter into the NcoI and BamHI restriction sites of plasmid pET11. PCR mutagenesis of the diphtheria toxin gene was employed to obtain an NcoI gene cassette that encoded 385 amino terminal residues of diphtheria toxin that included the entire ADP-ribosyltransferase catalytic domain and the contiguous proximal portion of the toxin that is associated with translocation across cellular membranes. The mutagenesis of the native diphtheria toxin gene also resulted in the deletion of the coding region for the native toxin binding domain, introduction of coding sequences for a translation initiation ATG codon, a seven residue linker segment for fusion with the *Gmcsf* gene, and convenient flanking NcoI restriction enzyme sites for cloning. The diphtheria toxin gene PCR mutagenesis primers included a 5' primer (5'-GCCATGGGCGCTGATGATGTTGTTGATTC-3') introducing an NcoI restriction enzyme site and ATG codon, and a 3' primer (5' GCCATGGAGCCACCTCCACCCGATTTATGCCCCGGAGAATACGC-3') incorporating sequences encoding a linker domain for steric spacing of the murine GMCSF gene and an NcoI restriction enzyme site. The expression plasmid pET11dDT-mGMCSF was constructed by the cloning of the intact DT NcoI gene cassette into the NcoI site of pET11d-GMCSF as shown in Figure 6. Cloning strategies and other genetic manipulations were positioned to assure maintenance of the translational reading frame, and fidelity of PCR amplification and genetic constructions were confirmed by DNA sequencing. Oligonucleotide primers were synthesized with an Applied Biosystems 394 DNA synthesizer at the University of Minnesota Microchemical Facility. Plasmid DNAs were prepared by use of the Wizard DNA purification resin (Promega, Madison, WI). DNA fragments amplified by the polymerase chain reaction (PCR) were initially cloned into the pT7Blue vector as directed by the manufacturer (Novagen), with DNA sequencing confirmation by the dideoxy method of Sanger using CircumVent thermal

cycling reagents (New England Biolabs, Beverly, MA). Restriction endonucleases, Taq DNA polymerase, and T4 DNA ligase were procured from BRL-Life Technologies (Gaithersburg, MD), Promega, New England Biolabs, or Perkin Elmer (Norwalk, CT), and used according to the specifications directed by the manufacturer. Standard techniques were employed for other manipulations of DNA including agarose gel electrophoresis, isolation and purification of restriction endonuclease fragments, cloning, and plasmid transformation into bacteria.

Expression and Purification of the Recombinant Fusion Toxin DT_{ct}GM-CSF.
All manipulations of *E. coli* bearing intact recombinant fusion toxin were performed under modified Biosafety Level 3 (BL3) containment practices. Initial attempts to achieve high level expression using techniques in Dr. Perentesis' laboratory that were developed for other fusion toxins resulted in production of relatively low yields for DT_{ct}GM-CSF because of product accumulation in inclusion bodies and aggregation. He an his colleagues subsequently successfully developed a refined production and expression method to isolate the product from bacterial inclusion bodies that yields high quality purified recombinant DT_{ct}GM-CSF. These refined methods are detailed in Figure 8, and include the addition of inclusion body isolation and protein recovery and renaturation procedures. In summary, *E. coli* HMS174(de3)plysS is transformed with pET11d:DT-mGMCSF and grown at 37 °C in LB medium with carbenicillin (50 μg/ml) to an absorbance (Å595) of 0.55-0.65. Expression of the fusion gene is induced by the addition of isopropyl-B-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The bacterial cells are then collected by centrifugation after one hour of induction. The bacterial pellets are resuspended in TE buffer (50mM Tris/20mM EDTA/100mMNaCl pH 7.8). Lysis of the cells is achieved by adding 5 mg/ml of lysozyme and incubating 30 min in 40 °C. The insoluble extract containing is resuspended in Triton-X buffer (89%TE buffer/11%vol/vol Triton-X) and homogenized briefly with a tissumizer. After incubation at room temperature for 1 hour, the inclusion bodies are obtained by ultracentrifugation at 24,000 g for 50 min and solubilized in solubilization buffer (7M guanidine/0.1M Tris pH:8/2mM EDTA/65M dithioerythriotol) overnight at room temperature. The solubilized protein is collected by ultracentrifuge at 40,000g for 10 min, then diluted 100 times in refolding buffer (0.1M Tris pH:8/0.5M L-arginine/0.9mM Oxidized glutathione/2mM EDTA/0.1M Urea) for 48 hours at 100 °C. The refolded protein is diafiltrated and ultrafiltrated against 20mM Tris pH:7.8/100mM Urea, then loaded on to Q-sepharose column and eluted with 0.3M NaCl in 20mM Tris pH:7.8. The eluted protein is diluted 5 times with 20mM Tris, then loaded on to Q-sepharose column again and eluted with linear salt gradient from 0.06-0.4M NaCl in 20mM Tris pH:7.8. Final size-exclusion purification is conducted using a TSK-gel G2000 column.

In Vitro Characterization of Recombinant DT_{ct}GM-CSF. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses using equine diphtheria antitoxin (anti-DT; Bethyl) antibodies were performed by standard methods using 10% gels in a Mini-Protean II gel apparatus (Bio-Rad). Diphtheria toxin standards were obtained from Sigma. Primary antibodies were used at a dilution of 1:5000. Secondary antibodies, rabbit anti goat(Bethyl laboratories), covalently linked to horseradish peroxidase were used at a 1:5,000 dilution. SDS-polyacrylamide gel analysis of the uninduced and induced whole cell extract, Q-sepharose chromatography column fractions revealed high level production and >95% purity of a monomeric protein with a molecular mass of ~57 kDa, the expected molecular mass of DT_{ct}GM-

CSF as deduced from its nucleic acid sequence. The final product of TSK-gel G2000 purification is estimated to exceed 98% purity (Figure 8). The integrity of expression of both the diphtheria toxin and GM-CSF moieties of DT_{ct}GM-CSF was confirmed in immunoblot analysis employing antisera to diphtheria toxin (Figure 9).

Plans for the Next Year of Funding. In year 2, we plan to continue and complete the studies of DT_{ct}GM-CSF in *in vitro* models of hematopoiesis. These studies will include experiments to inhibit the CFU-GM colony formation in methylcellulose cultures established from *Nf1+/+*, *Nf1+/-*, and *Nf1-/-* fetal liver cells, and of bone marrow cells of mice previously engrafted with fetal liver cells of all three *Nf1* genotypes. We plan to also initiate the *in vivo* treatment of *Nf1* and wild-type mice with DT-GMCSF that will extend through years 2-3 of this proposal. Groups of 5 wild-type mice will be treated with daily i.p. doses for 5 treatment days for a total of 1 μg, 2 μg, 5 μg, 10 μg, or 20 μg murine DT_{ct}GMCSF. These regimens were chosen since they were well tolerated and yielded very encouraging results in the preliminary studies using human DT_{ct}GMCSF. The dose-finding study will be expanded with either lower or higher dose ranges depending on the observed toxicity. In treatment regimens involving *Nf1* mice, the same numbers of *Nf1-/-* and *Nf1+/+* transplant recipients will be enrolled in phase 1 and phase 2 experiments and the clinical and laboratory observations will be identical. However, the phase 1 experiment will also involve treating a cohort of the mice that are homozygous for a mutation in the β chain of the GM-CSF receptor (GMRβ) with DT_{ct}GMCSF (Nishinakamura et al., 1995). Cells from these mice do not express the GM-CSF receptor, and should therefore be resistant to the toxic effects of DT_{ct}GMCSF. The Shannon laboratory has obtained these mice and has crossed them with *Nf1* mice. *GMRβ-/-* mice provide an excellent model for testing the specificity of DT_{ct}GMCSF therapy, and we predict that they will show no changes in blood cell counts even at high doses of the recombinant fusion toxin.

Technical Objective (Aim) 2: Chemotherapy and Radiation Studies

Overview. These studies are based upon clinical observations which suggested that children with NF1 are at increased risk of developing myeloid and other tumors after being treated with multi-modal therapy for another cancer (Maris et al., 1997). These human data implicated exposure to alkylating agents in the development of therapy-related leukemia (t-ML). Based on these clinical findingd and on the 10% risk of leukemia in untreated *Nf1+/-* mice (Jacks et al., 1994), we exposed *Nf1+/-* mice to mutagenic agents frequently used to treat malignancies in patients with and without NF1 (Mahgoub et al., 1999). Preliminary data from these studies were presented in our original application. We have now completed our analysis of this cohort as described in the appended article (Mahgoub et al., 1999). Initially, mice were enrolled from the inbred 129Sv strain in which the *Nf1* mutation was created. In order to perform LOH analysis at loci other than *Nf1* in alkylator-treated mice, F1 offspring from a cross between the 129Sv and C57BL6 strains were used in the latter part of the experiment. *Nf1+/-* and *Nf1+/+* littermates were assigned to observation (control group) or to receive 6 weeks of treatment with either etoposide or CY beginning at 6-10 weeks of age.

Myeloid leukemia or MPD developed in 4 of 101 *Nf1+/+* mice, 2 of which received CY (Table 1). In contrast, MPD was diagnosed in 14% of the untreated *Nf1+/-* mice (8 of 58), in

25% of the etoposide-treated animals (8 of 32), and in 43% (16 of 37) of the mice assigned to the CY group (Table 1). A Kaplan-Meier analysis demonstrated that the incidence of MPD was significantly higher and the latency period was reduced in CY-treated mice ($p = 0.001$ vs. untreated *Nf1*+/- mice), but not in the etoposide group ($p = 0.2$ vs. the untreated group). Both control and CY-treated 129Sv *Nf1*+/- mice showed higher rates of MPD than the corresponding groups of 129Sv x C57BL6 mice (Table 1). Most of the treated and control mice developed MPD with large numbers of mature neutrophils and monocytes in the peripheral blood. Bone marrow examination revealed an overwhelming predominance of myeloid cells with a shift toward immature elements, and sections of the spleen showed expansion of red pulp with infiltration of myeloid cells at various stages of differentiation admixed with areas of erythropoiesis. Frank myeloid leukemia was diagnosed in 2 of the mice (1 etoposide-treated and 1 CY-treated). LOH at *Nf1* correlated with clinical evidence of MPD in *Nf1*+/- mice and this invariably involved loss of the wild-type *Nf1* allele. Within the CY-treated group, leukemic cells from 129Sv x C57BL6 mice showed a much lower incidence of LOH than cells from 129Sv animals (Mahgoub et al., 1999).

Table 1
Incidence of Leukemia in *Nf1*+/- Mice

Strain and Treatment	Genotype	No. of Mice	No. (and %) with Leukemia
129Sv			
None	*Nf1*+/-	46	8 (17%)
Etoposide	*Nf1*+/-	32	8 (25%)
Cyclophosphamide	*Nf1*+/-	12	7 (58%)
129Sv x C57BL6			
None	*Nf1*+/-	12	0 (0%)
Cyclophosphamide	*Nf1*+/-	25	9 (36%)

This *in vivo* model of t-ML has a number of novel features that facilitate basic and translational research studies of this important clinical disorder. First, the fact that *Nf1* mice recapitulate clinical observations made in NF1 patients suggest that this model will be highly relevant for understanding specific aspects human t-ML. Second, *Nf1* provides a genetic target to examine the mechanism(s) of alkylator-induced DNA damage in hematopoietic cells. Finally, this model allows us to undertake controlled experiments that are neither feasible nor ethical in humans. We are exploiting this system to ask if radiation therapy, alone and in combination with CY, accelerates tumorigenesis in heterozygous *Nf1* mice. This question is highly relevant to the care of individuals with NF1 because radiation therapy is used frequently to treat NF1-associated tumors. Indeed, our initial data led the Children's Cancer Group to modify the treatment of children with NF1 who develop brain tumors so that they are not assigned to alkylator-intensive regimens. In addition to treating *Nf1* mutant mice with radiation and CY, we are performing molecular analyses at the *Nf1* locus and are examining the incidence of hypoxanthine guanine phosphoribosyl transferase *(Hprt)* mutations as an *in vivo* measure of DNA damage.

In year 1, the technical objectives related of this aspect of the project were (1) to breed and genotype mice for the proposed studies, and (2) to perform pilot studies of CY + radiation to establish a dose for treating mice. We have accomplished these goals and have begun enrolling the treatment groups.

Pilot Treatment of *Nf1*+/- Mice with CY and Irradiation. The mice treated with CY in our initial study were exposed to 100 mg/kg/week for 6 weeks. This dosing schedule was based upon data provided to us by Dr. Peter Houghton (St. Jude Children's Research Hospital) and on pilot data we generated in 129Sv mice. In the course of the experiment, we noticed that many F1 129Sv/C57BL6 mice maintained normal neutrophil counts (data not shown). This resistance to neutropenia seen in the 129Sv/C57BL6 mice may explain the lower incidence of MPD seen in this genetic background (Table 1). We therefore performed a dose escalation study to define the maximally tolerated dose of CY in 129Sv/C57BL6 mice and observed significant leukopenia that was associated with neutropenia, but with minimal morbidity, at 200 mg/kg/week for 6 weeks (Figure 10).

We next examined the feasibility of combining this dose of CY with total body irradiation in 129Sv x C57BL6 mice. In these studies, mice received a single fraction of either 2 or 3 Gray (200 or 300 rads) of total body irradiation as a single fraction two weeks after the last dose of CY. The use of total body irradiation insures that all of the blood-forming marrow is exposed, and previous data have shown that 2-3 Gy is more leukemogenic than higher or lower doses in susceptible mouse strains (Major and Mole, 1978). We found that *Nf1*+/- 129Sv x C57BL6 F1 mice tolerated these treatment regimens well with no deaths occurring during or after the radiation phase of the study. White blood cell counts are shown in Figure 11. Based on these results, we will compare CY + 3 cGy, with CY alone, irradiation alone, and no treatment as shown in Table 2. In order to minimize the number of animals treated, we plan to assign fewer mice to the control and CY alone arms (groups 1, 2, 5, and 6) because we have already ascertained the expected incidence of myeloid diseases in these cohorts. We began enrolling mice in July, 1999 and have entered 42 to date. An additional 37 mice have been genotyped and will begin treatment later this month.

Table 2
Treatment Schedule for F1 C57BL6/129Sv *Nf1*+/- and *Nf1*+/+ Mice

Group	Number	Genotype	Treatment	Schedule
1	15	*Nf1*+/-	None	Untreated Control
2	15	*Nf1*+/+	None	Untreated Control
3	30	*Nf1*+/-	Radiation	300 cGy x 1 dose
4	30	*Nf1*+/+	Radiation	300 cGy x 1 dose
5	15	*Nf1*+/-	CY	200 mg/kg/week i.p. x 6 weeks
6	15	*Nf1*+/+	CY	200 mg/kg/week i.p. x 6 weeks
7	30	*Nf1*+/-	CY + Radiation	200 mg/kg/week i.p. x 6 weeks, then 300 cGy x 1 dose
8	30	*Nf1*+/+	CY + Radiation	200 mg/kg/week i.p. x 6 weeks, then 300 cGy x 1 dose

Hprt Inactivation as a Surrogate Marker for Chemotherapy-Induced DNA Damage in Myeloid Cells Hypoxanthine guanine phosphoribosyl transferase (HPRT) is a cellular enzyme crucial in the metabolism of the chemotherapeutic agent thioguanine into deoxythioguanine triphosphate which can then be incorporated into DNA and cause cell death. Lymphocytes that have

inactivated *Hprt* acquire the ability to proliferate in the presence of 6-thioguanine because they are unable to convert this drug to its active metabolite. *Hprt* mutation rate has been used as a surrogate marker for DNA damage induced by mutagenic compounds such as CY and irradiation, and for evaluating potential chemoprotective compounds (Kataoka et al., 1996; Meng et al., 1998). However, it is not known if reducing the frequency of *Hprt* inactivation will correlate with a decrease in the risk of therapy-related cancer *in vivo.* If this proves true, *Hprt* could be used as a surrogate marker to test the mutagenic potential of new chemotherapeutic agents and of specific regimens. Our *Nf1* mouse model provides the first opportunity to rigorously address this important question. *Hprt* is an attractive marker for ascertaining mutagenic damage in this model because somatic inactivation of the relevant target genes *(Hprt* or *Nf1)* contributes to clonal proliferation in both instances.

We recently established the *Hprt* assay in our laboratory using published methods (Meng et al., 1998). *Hprt* mutation frequency is measured in male mice because this locus is on the X chromosome. We initiated these studies in C57BL6/129Sv mice treated with CY at a dose of 200 mg/kg/week for either 1 week (single dose) or for 6 weeks. The mice are sacrificed and splenocytes are isolated 55-60 days after the last drug dose. Preliminary data from a cohort of mice treated for 1 week showed a greater than 10 fold increase in the mutation frequency of CY-treated mice compared to controls (mutation frequency = 4.7×10^{-5} in CY-treated mice vs. 3.3×10^{-6} in the controls; $p = 0.038$).

In Vitro Transcription Translation (IVTT) Assay to Screen Murine Tissues for *Nf1* Mutatons. Most of the mutations that cause NF1 involve nucleotide substitutions or small deletions/insertions that lead to premature termination of protein translation (Upadhyaya et al., 1994). Heim et al. (Heim et al., 1995; Heim et al., 1994) described an IVTT assay to screen human patient samples for mutations in *NF1* which we used successfully to define mutations in childhood leukemias (Side et al., 1998; Side et al., 1997). We have now developed oligonucleotide primers and amplification conditions for screening murine *Nf1* by IVTT (data not shown). This assay will allow us to correlate the incidence and spectrum of somatic mutations that occur at the *Nf1* locus in murine tumors with treatment group (i.e. CY alone, radiation alone, or CY + radiation). These studies will allow us to test the hypothesis that alkylating agents and radiation inactivate target genes by distinct mechanisms.

Plan for the Next Year of Funding. Having achieved our goals of defining a treatment schedule and establishing an active breeding colony in year 1, we will now finish enrolling and treating the cohorts of mice shown in Table 2. We will observe these mice and will begin collecting follow-up data. We will also compare *Hprt* mutation rates in untreated mice and in mice treated with CY alone, radiation alone, or CY + radiation to test the hypothesis that *Hprt* mutation rates will predict survival and cancer rates. We will also investigate any murine leukemias or solid tumors for LOH at *Nf1,* and will perform IVTT to define the incidence and nature of somatic *Nf1* mutations in samples that do not show LOH.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Developed breeding stocks of mouse strains and generated recipients repopulated with *Nf1-/-* or wild-type fetal liver cells.

- Completed preclinical study of MM dosing in wild type mice and selected drug dose for preclinical efficacy study.
- Performed preclinical evaluation of MM in transplant recipients.
- Initiated correlative biochemical studies of tissues from MM-treated mice.
- Developed strategy and construct for making DT_{ct}GM-CSF, and produced sufficient amounts of this compound for *in vitro* and *in vivo* testing.
- Performed preclinical studies to determine optimal regimen for administering cyclophosphamide with irradiation, and enrolled initial cohorts of mice.

(8) REPORTABLE OUTCOMES

(a) Review Article

Weiss B, Bollag G, Shannon KM. Hyperactive Ras as a therapeutic target in neurofibromatosis type 1. *Am J Med Genet* 1999; **89:** 14-22.

(b) Model Development

The studies conducted to date have established a regimen for administering cyclophosphamide with and without radiation to F1 C56BL6/129Sv mice that should be useful for future studies of tumorigenesis and chemopreventive strategies in *Nf1* mice.

(c) Employment and Research Opportunities

Richard Chao, M.D. is a fellow in adult hematology/oncology who is supported by this award. Dr. Chao is primarily working on the radiation/cyclophosphamide studies. He is interested in pursuing a career in translational research.

Brian Weiss, M.D. is a fellow in pediatric hematology/oncology who is participating in the experimental therapeutics studies. His salary is supported by a training fellowship from the Frank A. Campini Foundation.

Alfrd Au, Charles Fezzie, Zabi Wardak, Myla Sanchez Vikas Arora, and Abigail Peterson are technical personnel in the investigator's laboratories who have received partial salary support from this award.

(9) CONCLUSIONS

Our progress on each Technical Objective with plans for the duration of this award are presented in detail in the Body. The nature of translational research is that it involves considerable effort in generating reagents, performing the experiments, and obtaining long-term follow-up. These studies are proceeded well to date and are on or ahead of schedule. We tentatively conclude that MM is unlikely to provide benefit in treating tumors that arise in individuals with NF1.

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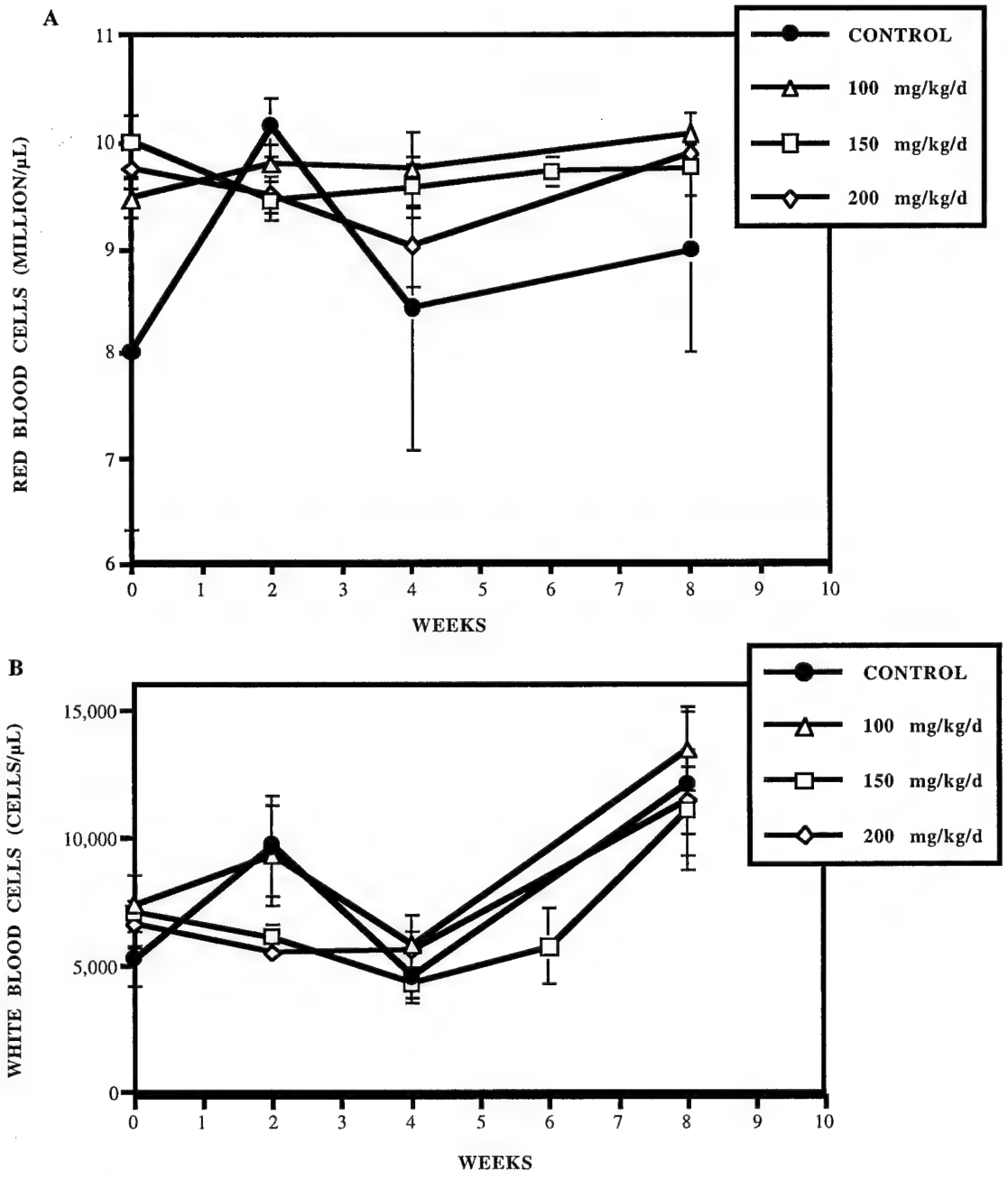


Figure 1. Red blood cell (panel A) and white blood cell counts (panel B) in wild type mice treated with 100-200 mg/kg /day of MM (n = 5 in each group).

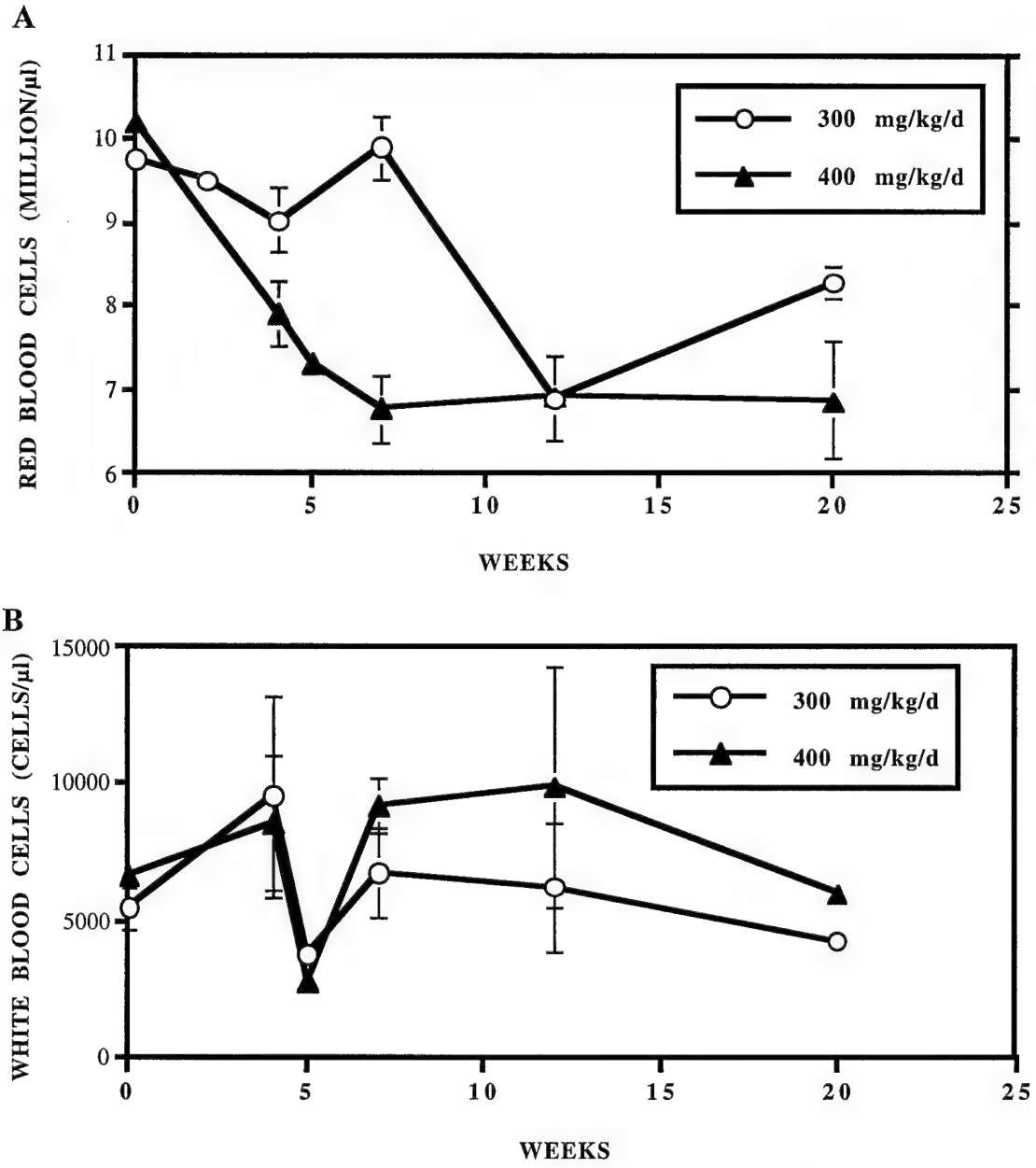


Figure 2 Red blood cell (panel A) and white blood cell counts (panel B) in wild type mice treated with 300 or 400 mg/kg /day of MM for 20 weeks (n = 5 in each group). Hemoglobin and hematocrit values declined in parallel with RBC counts.

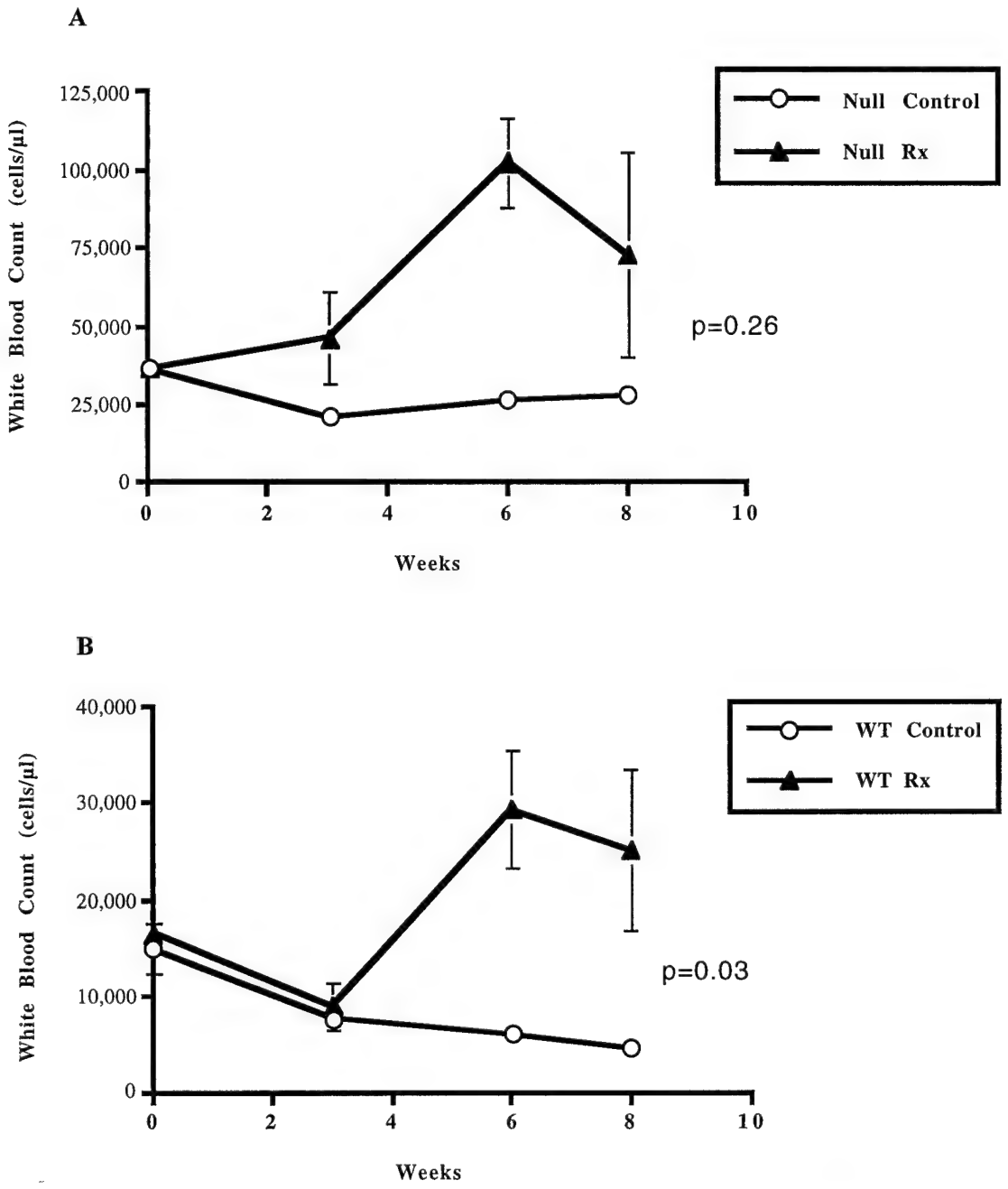


Figure 3. White blood cell counts in recipient mice engrafted with *Nf1-/-* fetal liver cells (panel A) or with wild type fetal liver cells (panel B). Control mice were not treated and "Rx" mice received MM at a dose of 400 mg/kg/day . Note that the baseline white blood cell counts were much higher in recipients of *Nf1-/-* cells. The increase in white blood cell counts was due to a rise in myeloid cells in both groups of Rx mice (n = 7-10 in each group).

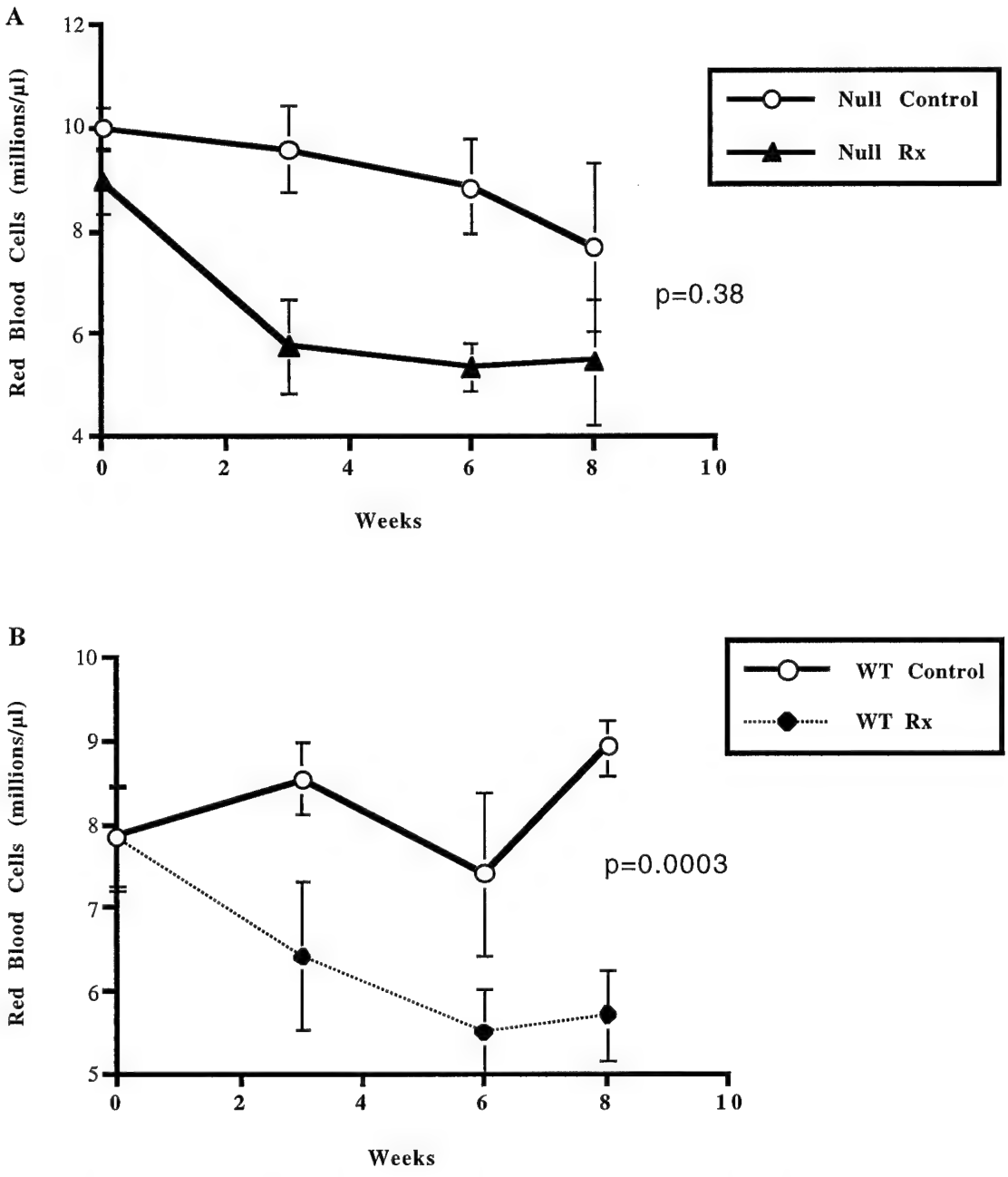


Figure 4. Red blood cell counts in recipient mice engrafted with *Nf1-/-* fetal liver cells (panel A) or with wild type fetal liver cells (panel B). Control mice were not treated and "Rx" mice received MM at a dose of 400 mg/kg/day (n = 7-10 in each group).

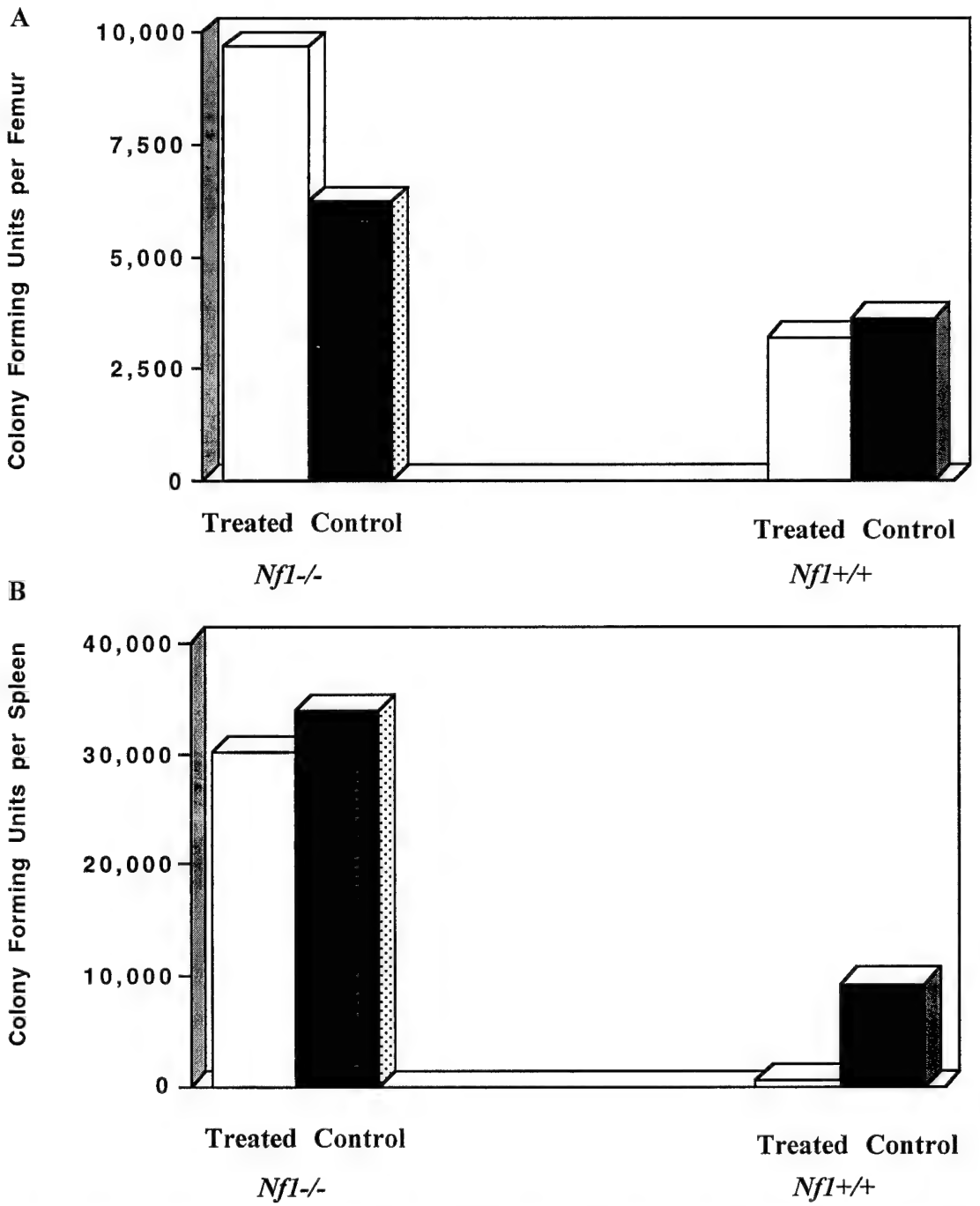
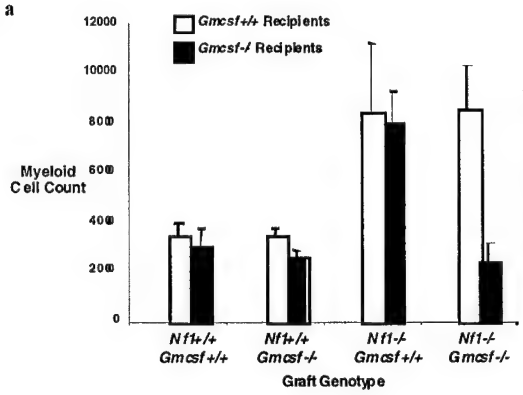


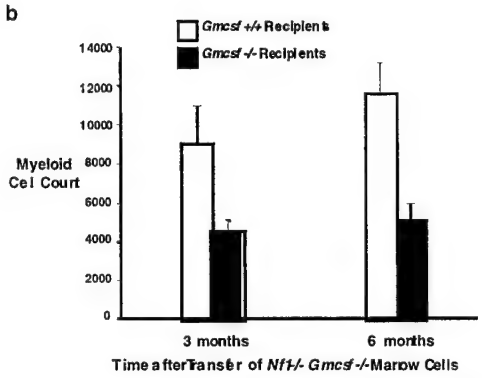
Figure 5. Total numbers of CFU-GM progenitor colonies enumerated in the femurs (panel A) and spleens (panel B) in recipients of *Nf1-/-* or wild type fetal liver cells. Note that the baseline numbers of CFU-GM is markedly expanded in *Nf1-/-* recipients at baseline. (n = 4 in *Nf1-/-* recipients, and n = 6 in wild type recipients).

Figure 6. Influence of *Nf1* and *Gmcsf* Genotype on Myeloid Cell Counts in Primary and Secondary Recipients



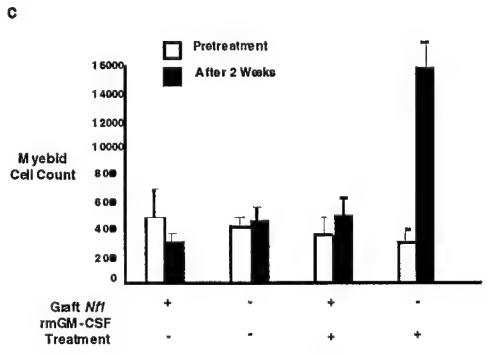
The MPD induced by adoptive transfer of *Nf1* null cells is attenuated by the lack of GM-CSF in primary recipients.

Myeloid cell counts (± SEM) three months after adoptive transfer in surviving mice engrafted with donor fetal liver cells (n = 75). *Gmcsf-/-* recipients of *Nf1-/- Gmcsf-/-* grafts showed lower myeloid cell counts than recipients in which either host ($p<0.01$) or graft ($p<0.005$) could produce GM-CSF.



The MPD induced by the adoptive transfer of *Nf1 -/-* cells is attenuated by the lack of GM-CSF in secondary recipients.

Ten pairs of irradiated secondary wild type (open bars) and *Gmcsf-/-* (closed bars) recipients received the same bone marrow cells. Myeloid cell counts (± SEM) were elevated in wild type versus *Gmcsf-/-*hosts 3 and 6 months later ($p<0.05$ and $p<0.005$, respectively). The myeloid cell counts of the *Gmcsf-/-* recipients are in the normal range for wild type mice.



The MPD can be re-established by treatment with sub-therapeutic doses of GM-CSF in recipients of *Nf1-/- Gmcsf-/-* grafts.

Myeloid cell counts (± SEM) in rmGM-CSF-treated and control recipients immediately prior to and after 2 weeks of treatment with rmGM-CSF. Recipients of *Nf1-/- Gmcsf-/-* grafts that received rmGM-CSF had elevated myeloid counts when compared to all controls ($p<0.01$).

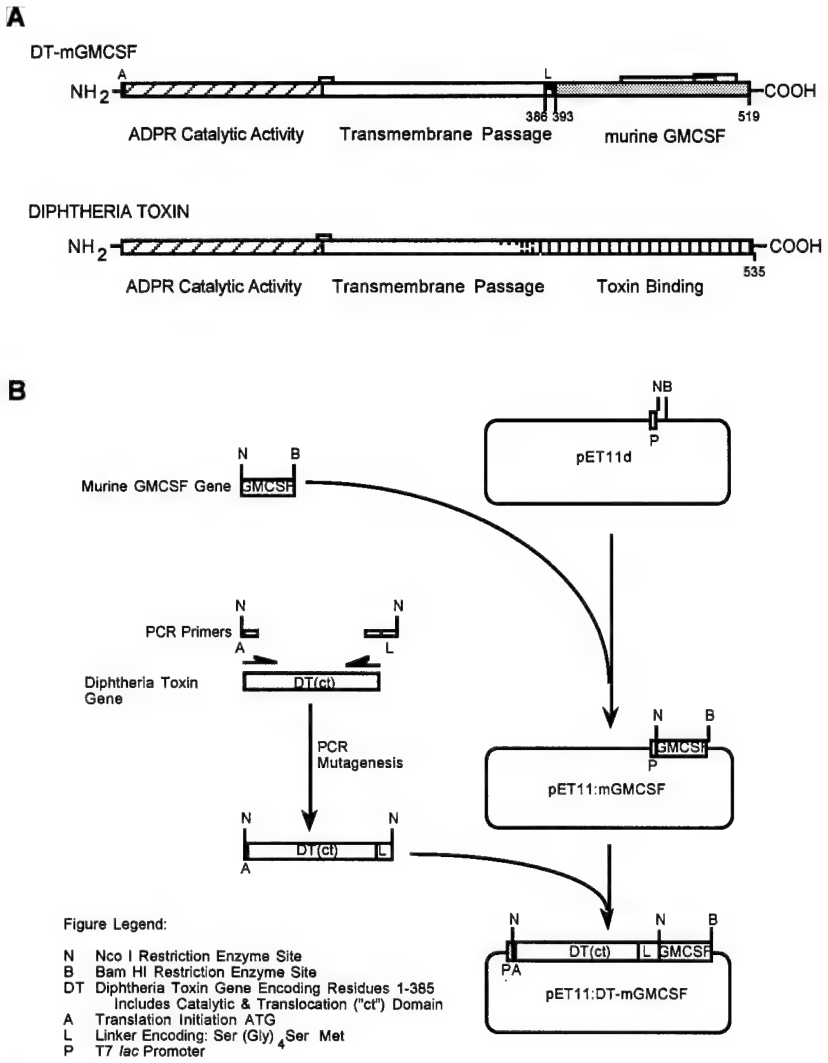


Figure 7. (A) Structure of the DT_{ct}GM-CSF fusion toxin and comparison to diphtheria toxin. The native receptor-binding domain of diphtheria toxin was genetically deleted and replaced with murine GM-CSF, separated by a short linking sequence ("L": Ser-(Gly)4-Ser-Met). The genetic addition of an ATG codon was used to introduce a methionine residue to the amino terminus of the fusion toxin ("A" = Met). (B) Construction of the recombinant growth factor - toxin fusion expression vector pET11d-DT-mGMCSF. A synthetic cDNA encoding mature murine GMCSF was cloned between the NcoI and BamHI sites of plasmid pET11d downstream of the T7 promoter to produce pET11d-GMCSF. PCR mutagenesis of the diphtheria toxin gene was employed to obtain a NcoI DT gene cassette that included: (i) the addition of an ATG methionine translation initiation codon immediately 5' of the initial GGC glycine codon of mature native diphtheria toxin, (ii) a short 3' linker sequence encoding seven amino acid [Ser-(Gly)4-Ser-Met] residues downstream of diphtheria toxin lysine residue 385, and (iii) flanking NcoI restriction endonuclease sites. Expression plasmid pET11dDT-mGMCSF was constructed by the cloning of the intact DT NcoI gene cassette into the NcoI site of pET11d-mGMCSF.

DT$_{ct}$GM-CSF EXPRESSION AND PURIFICATION

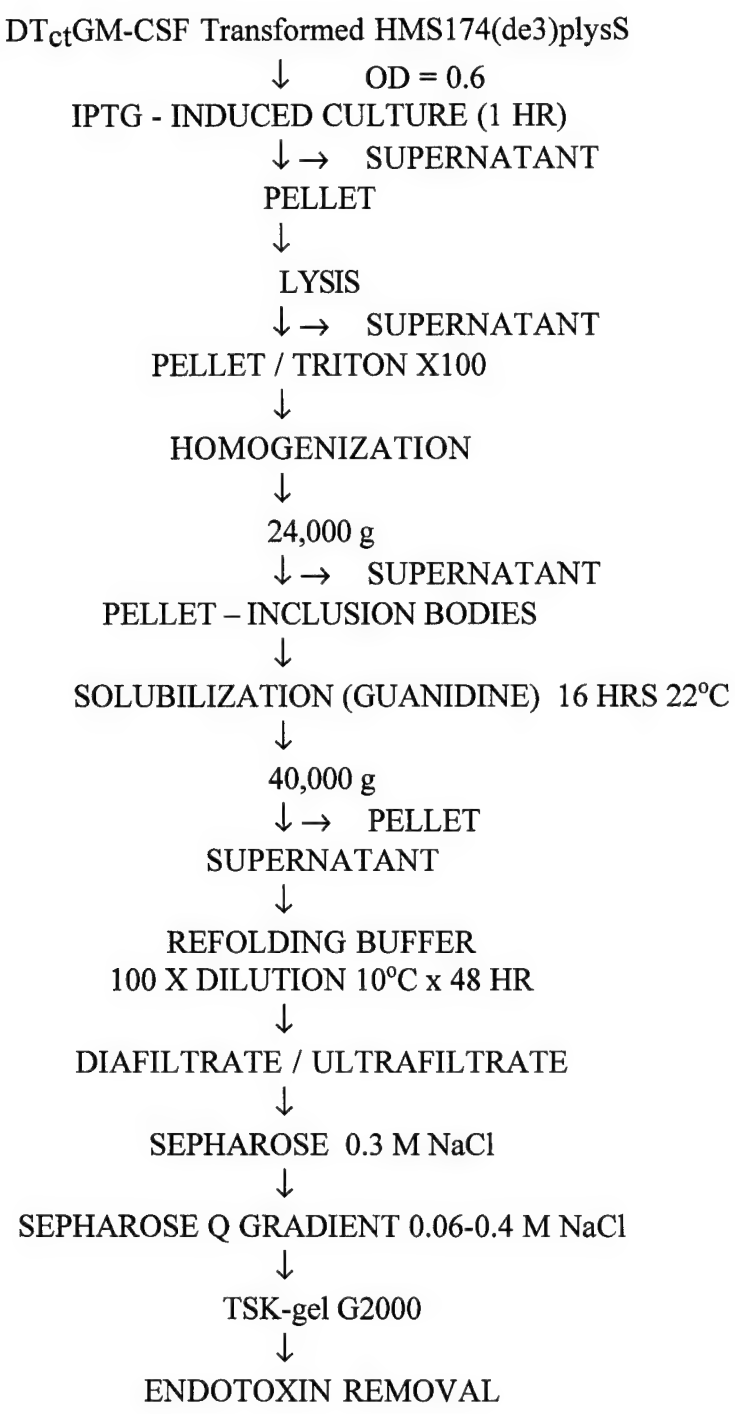


Figure 8. Expression and purification of the recombinant DT$_{ct}$GM-CSF fusion toxin (see text for details).

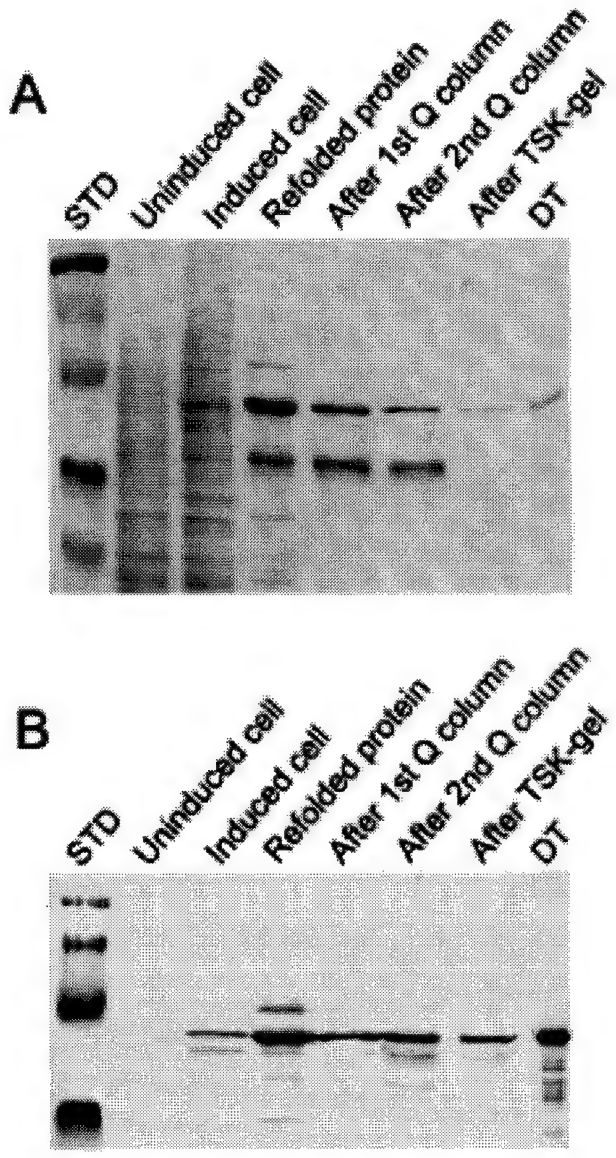


Figure 9. Purification of recombinant DT_{ct}GM-CSF . The expression and sequential Q sepharose columns, TSK-gel G2000 column purification of DT_{ct}GM-CSF from IPTG-induced 500ml cultures of E. coli were analyzed by SDS-PAGE (A), and anti-DT immunoblots (B), detecting the 58 kDa DT-mGMCSF protein. The migration of prestained molecular weight size standards is indicated, and for immunoblot analysis, native DT was used as controls.

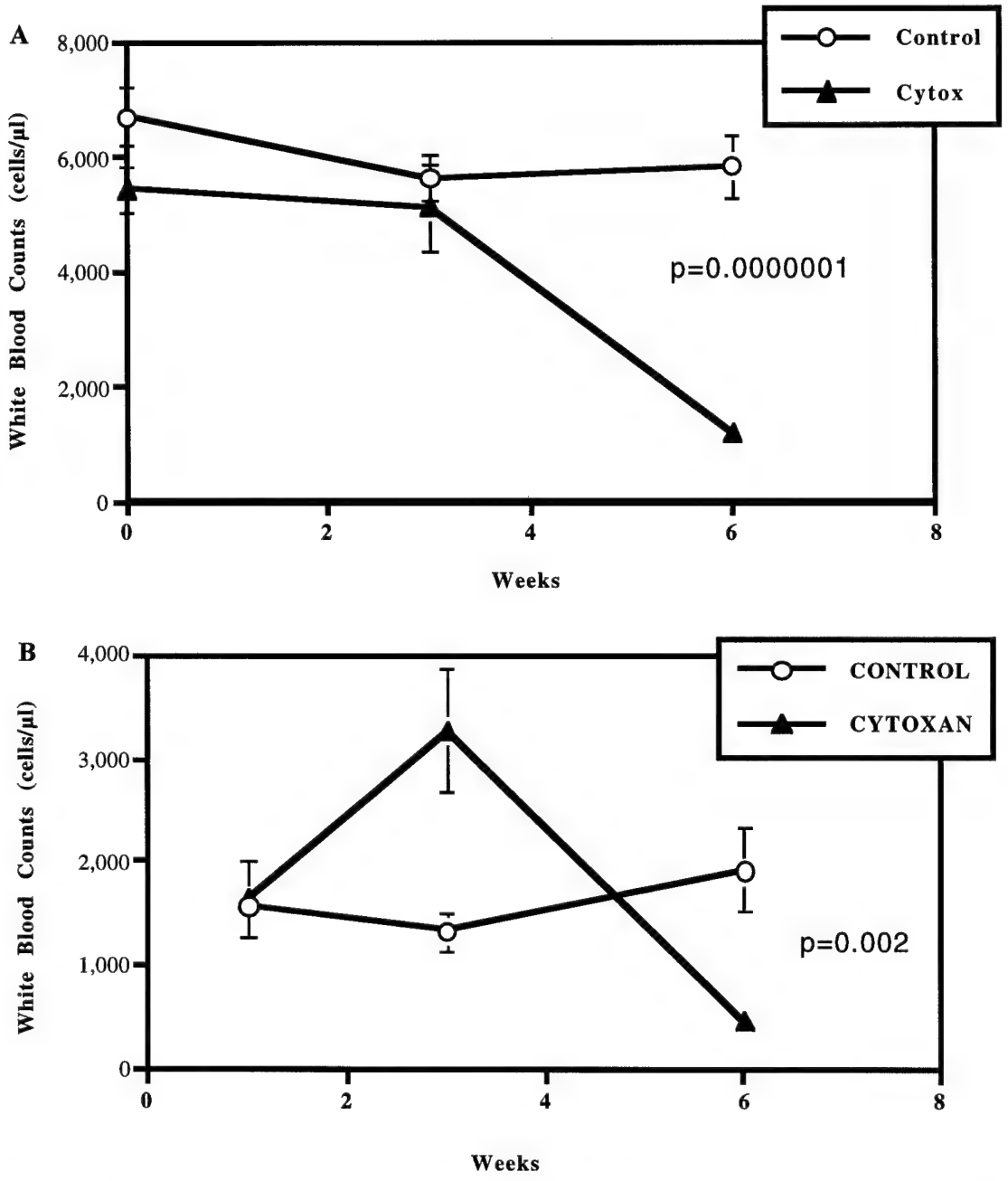


Figure 10. Total white blood cell counts (panel A) and blood neutrophil counts (panel B) in untreated control mice and in mice that received 200 mg/kg/week of cyclophosphamide for 6 weeks (n = 20 in each group).

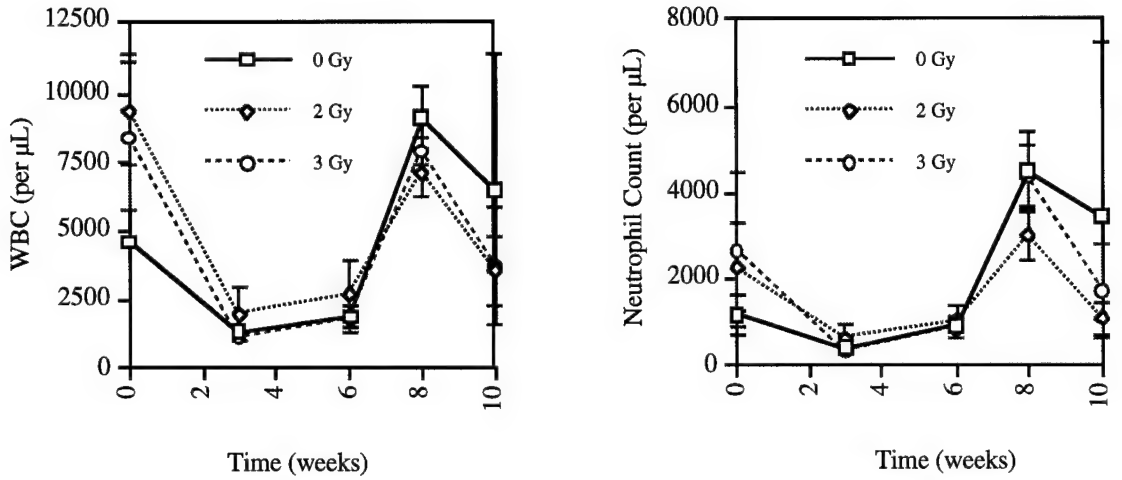


Figure 11. Total white blood cell counts (left panel) and blood neutrophil counts (right panel) in mice that received 200 mg/kg/week of cyclophosphamide for 6 weeks followed by either no radiation, 2 Gy, or 3 Gy (n =15).

AMERICAN JOURNAL OF MEDICAL GENETICS (SEMIN. MED. GENET.) 89:14–22 (1999)

ARTICLES

Hyperactive Ras as a Therapeutic Target in Neurofibromatosis Type 1

BRIAN WEISS, GIDEON BOLLAG, AND KEVIN SHANNON*

The *NF1* gene encodes neurofibromin, a GTPase-activating protein (GAP) for members of the $p21^{ras}$ (Ras) family, which negatively regulates Ras output by accelerating the conversion of active Ras·GTP to inactive Ras·GDP. Analysis of tumors from patients with neurofibromatosis type 1 (NF1) has shown biochemical evidence of hyperactive Ras as well as frequent loss of the normal *NF1* allele, consistent with its role as a tumor suppressor gene. Taken together, these data suggest that novel therapeutics directed against components of the Ras signaling cascade might provide effective treatments for certain pathological complications of NF1. Here we summarize data that support a role for hyperactive Ras in NF1 disease, including Ras processing, activation, and down-regulation. We review targets for rational drug design, provide preliminary results, and discuss implications for future studies. Am. J. Med. Genet. (Semin. Med. Genet.) 89:14–22, 1999. © **1999 Wiley-Liss, Inc.**

KEY WORDS: neurofibromatosis type 1; experimental therapeutics; Ras

INTRODUCTION

Before the gene for neurofibromatosis type 1 (NF1) was discovered, the cancer predisposition and propensity to develop multiple cutaneous neurofibromas in affected individuals suggested that the disease gene encoded a protein that negatively regulated cell growth. Furthermore, the autosomal dominant inheritance pattern of NF1 was consistent with the theory that *NF1* functions genetically as a tumor suppressor gene (TSG) in certain tissues. Both of these hypotheses could be tested only after the gene was identified in 1990 [Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990]. At that time, a segment of *NF1* cDNA unexpectedly showed strong homology with yeast and mammalian GTPase-activating proteins (GAPs) for members of the $p21^{ras}$ (Ras) family [Buchberg et al., 1990; Xu et al., 1990].

Ras proteins play a central role in cellular growth and differentiation. Ras output is tightly regulated by cycling between an active GTP-bound conformation (Ras·GTP) and an inactive GDP-bound state (Ras·GDP) [reviewed in Bourne et al., 1990; Wittinghofer, 1998]. Ras has a slow intrinsic GTPase activity that is enhanced by GAPs. These proteins greatly increase the rate of GTP hydrolysis and thereby act as negative regulators of Ras output [reviewed in Boguski and McCormick, 1993; Bernards, 1995; Wittinghofer, 1998]. Oncogenic *RAS* alleles carry single point mutations at the Gly12, Gly13, or Gln61 positions that greatly reduce the intrinsic GTPase activity and render these proteins resistant to GAPs [reviewed in Wittinghofer, 1998].

Ras proteins regulate cell fates by transducing signals from the plasma membrane to the nucleus via a series of downstream effectors [reviewed in Wittinghofer, 1998]. Ras·GTP recruits Raf kinase to the membrane, where its kinase activity is effective. Raf, in turn, activates a kinase cascade involving MEK kinase and the Erk1 and Erk2 isoforms of mitogen-activated protein (MAP) kinase (Fig. 1). The activation states of the phosphoinositol-3′-kinase (PI3K) and Rac/Rho pathways are also regulated by Ras·GTP in many cell types. The consequences of Ras activation are influenced by the cellular context and by cross talk between its downstream effectors.

Neurofibromin, the product of *NF1*, has a putative GAP-related domain (GRD); biochemical analysis of recombinant peptides corresponding to its GRD have demonstrated that neurofibromin is an authentic GAP for Ras [reviewed in Boguski and McCormick, 1993; Bernards, 1995]. Yeast strains with mutations in the *NF1* homologues *ira1* and *ira2* are susceptible to heat shock owing to an inability to down-regulate *ras1*. The observation that expressing the neurofibromin GRD in these yeast strains could restore a normal heat-shock response provided direct in vivo evidence supporting its role as a GAP for Ras [Ballester et al., 1990]. Furthermore, structural analysis of crystals containing a fragment of neurofibromin have shown a homologous "arginine finger" within the GAP domain

Brian Weiss is a fellow in pediatric hematology/oncology at the University of California, San Francisco (UCSF). His research focuses on translational studies of myeloid leukemia in *NF1* mice. Gideon Bollag is research director for small molecule therapeutics at Onyx Pharmaceuticals. His research involves exploiting signal transduction pathways for the development of cancer therapeutics. Kevin Shannon is in the Department of Pediatrics and is leader of the program in hematopoietic malignancies at UCSF. His laboratory studies inherited predispositions to myeloid leukemia.

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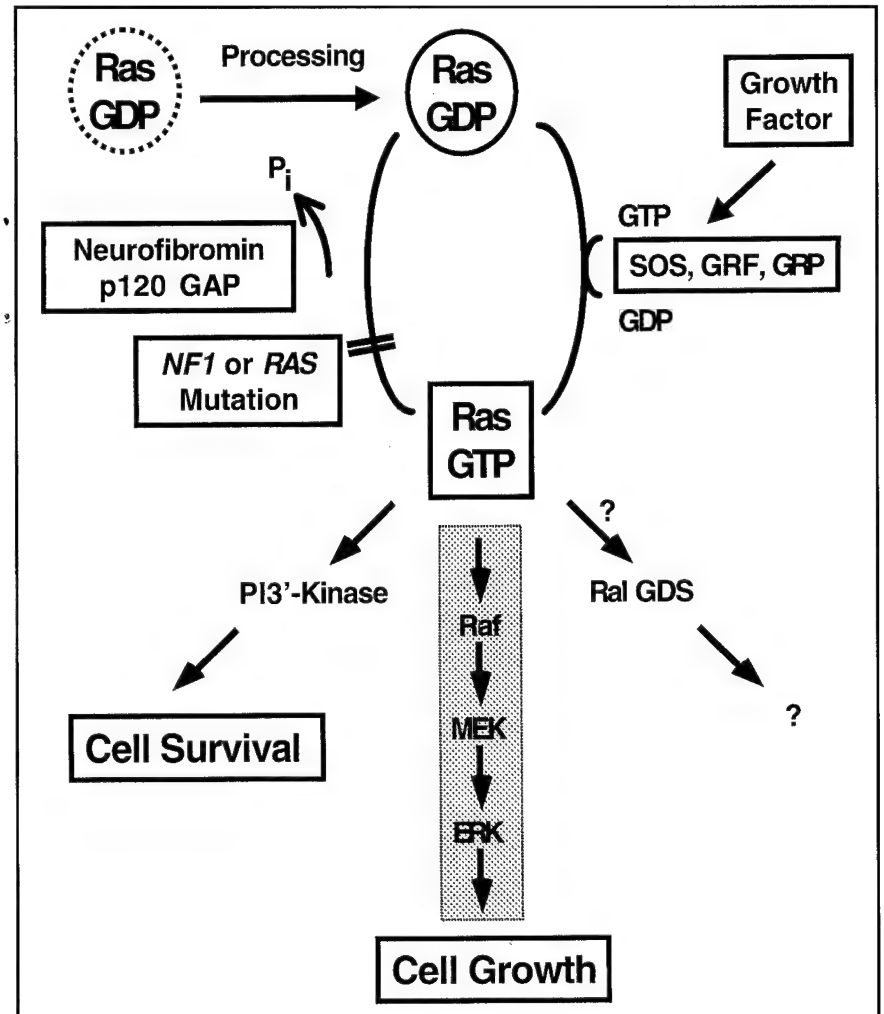

Figure 1. Overview of Ras activation by GEFs and signaling through downstream effectors. Ras cycles between an active GTP-bound conformation (Ras·GTP) and an inactive GDP-bound state (Ras·GDP). Growth factors induce cell growth, in part by activating the GEFs Sos, GRF, and GRP that bind Ras and stimulate nucleotide dissociation. Nucleotide exchange increases the percentage of Ras·GTP because the intracellular concentration of free GTP vastly exceeds that of GDP. Signaling terminates when Ras·GTP is hydrolyzed to Ras·GDP. Neurofibromin and p120 GAP regulate this process by accelerating the conversion of Ras·GTP to Ras·GDP. Oncogenic *RAS* mutations or inactivation of *NF1* perturb Ras signaling by favoring the GTP-bound state, as described in the text. Ras·GTP activation of the Raf/MEK/ERK kinase cascade stimulates proliferation in many cell types, and activation of the PI3K pathway has been shown to promote cellular survival. Ras interacts with other downstream effectors, such as Ral-GDS; all of the components of this pathway remain to be defined.

[Scheffzek et al., 1998]. Recently, structural analysis of crystals containing a fragment of p120 GAP in complex with Ras identified an arginine finger within the GAP domain that comes into close proximity to Gly12 and Gln61 and stabilizes a transition state between Ras·GTP and Ras·GDP [Scheffzek et al., 1997].

The GAP activity of neurofibromin has profound implications both for understanding the pathological complications of NF1 and for designing rational therapies. Studies of anti-Ras therapeutics are broadly applicable because *RAS* is the most common oncogene mutated in human malignancies. This review summarizes data that support a role for hyperactive Ras in NF1 disease, including Ras processing, activation, and down-regulation. It explores targets for rational drug design and preliminary results and presents implications for future studies.

HYPERACTIVE RAS IN NF1 PATHOLOGICAL MANIFESTIONS

Cells derived from patients with NF1 and *Nf1* knockout mice have been used to test the hypothesis that *NF1* functions as a TSG in mammalian cells and to determine if loss of function is associated with hyperactive Ras. Individuals with NF1 are predisposed to specific cancers, including malignant peripheral nerve sheath tumor (MPNST), astrocytoma, pheochromocytoma, and juvenile myelomonocytic leukemia (JMML). Somatic loss of constitutional heterozygosity (LOH), a hallmark of DNA segments that harbor TSGs, has been detected at *NF1* in MPNST, pheochromocytoma, and JMML [Skuse et al., 1989; Glover et al., 1991; Xu et al., 1992; Shannon et al., 1994]. In patients with familial NF1, LOH has invariably been associated with loss of the allele inherited from the unaffected parent. A subset of cutaneous neurofibromas also shows LOH at *NF1* [Colman et al., 1995]. Homozygous inactivation of *NF1* has been demonstrated directly in a case of MPNST, in a neurofibroma, and in a number of leukemias from children with NF1 [Legius et al., 1993; Sawada et al., 1996; Side et al., 1997]. Similarly, heterozygous *Nf1* knockout mice are predisposed to pheochromocytoma and myeloid leukemia, and these neoplasms show loss of the wild-type *Nf1* allele. Together, these human and murine data demonstrate that *NF1* functions as a TSG in at least a subset of tumors.

A number of observations support the idea that inactivation of *NF1* is associated with hyperactive Ras in tumor cells. MPNST cell lines derived from

The GAP activity of neurofibromin has profound implications both for understanding the pathological complications of NF1 and for designing rational therapies.

patients with NF1 show decreased in vitro GAP activity and markedly elevated levels of Ras·GTP [Basu et al., 1992; DeClue et al., 1992]. Increased Ras·GTP levels have also been reported in primary MPNSTs removed from patients with NF1 [Guha et al., 1996] and in Schwann cells isolated from homozygous *Nf1*-deficient embryos [Kim et al., 1995].

The role of neurofibromin in myeloid growth control has been studied extensively in humans and in mice. In a large series of children with JMML and related disorders, activating *RAS* mutations were detected only in bone marrow samples from patients without NF1, suggesting that inactivation of *NF1* and somatic *RAS* point mutations are functionally equivalent [Kalra et al., 1994]. In addition, leukemic cells from children with NF1 show a reduction in neurofibromin-specific GAP activity, elevated levels of Ras·GTP, and activation of MAP kinase [Bollag et al., 1996]. JMML cells form excessive numbers of hematopoietic progenitor colonies in cultures containing low concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF) [Emanuel et al., 1996].

Although homozygous mutant *Nf1* murine embryos die in utero of complex cardiac defects, fetal hematopoiesis is normal [Brannan et al., 1994; Jacks et al., 1994]. Like JMML cells, *Nf1*−/− fetal liver cells are hypersensitive to GM-CSF in culture [Bollag et al., 1996; Largaespada et al., 1996], and adoptive transfer into irradiated recipient mice induces a JMML-like disorder [Largaespada et al., 1996]. Unstimulated *Nf1*−/− hematopoietic cells demonstrate constitutive MAP kinase activity and hyperactivation in response to GM-CSF and other hematopoietic growth factors [Zhang, 1998]. Finally, *Myb*-transformed hematopoietic cell lines prepared from *Nf1*−/− fetal livers show an exaggerated and prolonged increase in Ras·GTP levels in response to GM-CSF [Largaespada et al., 1996]. Taken together, these human and murine studies strongly support a model whereby the tumor suppressor function of *NF1* is mediated through the ability of neurofibromin to down-regulate Ras output in immature myeloid and neural crest cells. Furthermore, the hypersensitivity of *Nf1*-deficient hematopoietic cells to GM-CSF and of *Nf1*−/− fetal neurons to neurotrophins (Vogel et al., 1995) implicates abnormal responses to growth factors that activate Ras in the excessive deregulated proliferation of these cells.

Although most *NF1* mutations truncate neurofibromin and are thought to function as null alleles, a few families with missense mutations have been reported. In one kindred, a missense mutation at a highly conserved residue in the GAP domain (Lys-1423) was not associated with an unusual phenotype [Li et al., 1992]. Recently, a mother and two sons with NF1 were identified in whom the disease was inherited with a novel missense mutation of Arg-1276 (the arginine finger) [Klose et al., 1998]. This mutation does not impair secondary or tertiary protein structure, does not reduce neurofibromin levels, and does not significantly influence neurofibromin binding to Ras; however, as predicted from the structural analysis, this mutation completely disables GAP activity. Most affected family members have café au lait spots, freckling, and scoliosis, and both children have a learning disability. The proband developed mutliple schwannomas, one of which underwent malignant degeneration [Klose et al. 1998]. The example of this family suggests that many of the clinical phenotypes associated with NF1 disease can be accounted for by a selective inability of neurofibromin to interact with and down-regulate Ras output and illustrates how molecular analysis of human patients can enhance our understanding of disease pathobiology.

A number of observations support the idea that inactivation of NF1 is associated with hyperactive Ras in tumor cells.

While all of these observations are consistent with the idea that *NF1* negatively regulates cell growth by accelerating GTP hydrolysis on Ras, this simple model does not explain other data [Boguski and McCormick, 1993; Bernards, 1995]. Acquired *RAS* mutations are uncommon in neural crest tumors, and overexpressing oncogenic *RAS* induces differentiation, rather than transformation, in some neural crest cell lines [Bar-Sagi and Feramisco, 1985; Noda et al., 1985]. Furthermore, neuroblastoma and melanoma cell lines that have inactivated both *NF1* alleles still show normal levels of Ras·GTP [Andersen et al., 1993; Johnson et al., 1993; The et al., 1993]. A conserved *Drosophila* homologue, with 60% homology to human *NF1*, is widely expressed at low amounts during all stages of pupal development [The et al., 1997]. Homozygous disruption of the *Drosophila NF1* gene produces viable and fertile mutants with a growth defect that is fully rescued by expression of a heat shock–inducible *NF1* transgene [The et al., 1997]. However, *Drosophila NF1* mutants do not demonstrate phenotypic abnormalities associated with Ras activation, and neither reducing nor increasing signaling through the Ras-Raf1 pathway affects the impaired growth phenotype. In contrast, stimulating the adenosine 3′5′-monophosphate (cAMP)-PKA pathway rescues the size defect in *NF1*-deficient pupae and restores a neuromuscular junction defect that is insensitive to Ras manipulations [Guo et al., 1997; The et al., 1997]. These data raise the possibility that *NF1* may negatively regulate growth by mechanism(s) that are independent of the level of Ras·GTP and that may involve the cAMP pathway in some cell types [Andersen et al., 1993; Johnson et al., 1993; The et al., 1993]. Involvement of the cAMP-PKA pathway in the neuromuscular junction phenotype in *Drosophilia* raises the possibility that some of the learning disabilities in individuals with NF1 and in *Nf1*+/− mice are not entirely due to hyperactive Ras.

A relevant question in considering therapeutic strategies is whether inacti-

vation of a single *NF1* allele (i.e., haplo-insufficiency) contributes to any of the complications of the disease. While few data speak to this question, it is possible that haplo-insufficiency at *NF1* induces subtle biochemical alterations that underlie some of the learning disabilities, pigmentary lesions, and tumors in affected individuals. Heterozygous *Nf1* mice have specific learning defects that are compatible with this idea [Silva et al., 1997]. In addition, recent data showing that some of the tumors that arise in heterozygous *p53* knockout mice retain a functional *p53* allele illustrate that TSGs may promote tumorigenesis by either homozygous inactivation (Knudson model) or dosage [Venkatachalam et al., 1998]. If some of the complications of NF1 develop in cells that retain a normal allele, therapies directed at enhancing gene expression represent a rational approach. This strategy is predicated on the observation that none of the NF1 mutations identified to date have been shown to encode proteins that function in a dominant negative manner.

A relevant question in considering therapeutic strategies is whether inactivation of a single NF1 allele (i.e., haplo-insufficiency) contributes to any of the complications of the disease.

RAS ACTIVATION BY GUANINE NUCLEOTIDE EXCHANGE FACTORS AND SIGNALING THROUGH DOWNSTREAM EFFECTORS

Because Ras is one of the most intensively studied proteins in modern biology, a great deal has been learned about Ras activation in response to extracellular stimuli. A number of the downstream targets of Ras·GTP are also known (Fig. 1). We summarize these processes briefly, since they provide a context for discussing approaches to rational drug design in NF1. Guanine nucleotide exchange factors (GEFs) bind to either Ras·GDP or Ras·GTP; this interaction results in guanine nucleotide dissociation from Ras followed by passive rebinding. Because the intracellular concentration of free GTP vastly exceeds that of GDP in cells, nucleotide exchange on Ras increases the percentage of Ras·GTP and enhances output. Signaling terminates when Ras·GTP is hydrolyzed to Ras·GDP. GAPs play an integral role in this process by stabilizing a transition state between Ras·GTP and Ras·GDP; this accelerates the t1/2 of the Ras GTPase from minutes to seconds. Structural analysis of Ras co-crystalized with the GRD of p120 GAP strongly implicates an arginine finger formed by Arg-1276 of neurofibromin as critical for this interaction [Klose et al., 1998]. As discussed earlier, a missense mutation at this codon was recently characterized in a family with NF1.

Three types of GEFs for Ras are currently known: Sos, GRF, and GRP. The mammalian Sos1 and Sos2 [Bowtell et al., 1992; Wei et al., 1992] were identified on the basis of homology to the *Drosophila* "son of sevenless" gene [Simon et al., 1991]. The mammalian GRF1 and GRF2 proteins [Cen et al., 1992; Martegani et al., 1992; Shou et al., 1992; Fam et al. ,1997] were identified as homologues of the budding yeast Cdc25 gene product [Broek et al., 1987; Robinson et al., 1987]. Ras GRP was found in a functional screen searching for proteins capable of transforming fibroblasts [Ebinu et al., 1998; Tognon et al., 1998].

Each of these GEFs is regulated in a different fashion. The Sos proteins are ubiquitously expressed and function downstream of tyrosine kinase growth factor receptors [Buday and Downward, 1993; Egan et al., 1993; Li et al., 1993; Rozakis-Adcock et al., 1993]. Activated tyrosine kinase growth factor receptors recruit adaptor proteins, such as Shc and Grb2, which interact through SH2 and SH3 domains and recruit Sos to the complex. This membrane localization of Sos results in activation of GEF function and, hence, GTP loading of Ras. The GRF proteins are tissue specific (primarily brain) and can be activated by both calcium [Farnsworth et al., 1995] and heterotrimeric G-protein-mediated phosphorylation [Mattingly and Macara, 1996]. Ras GRP expression is also limited, with the highest levels in lymphoid tissues and brain [Ebinu et al., 1998; Tognon et al., 1998]. This GEF contains calcium- and diacylglycerol-binding domains, suggesting that its activity may be regulated by phospholipase C.

Effector pathways downstream of Ras appear to be numerous and complementary (Fig. 1). Since the discovery that Raf kinase binds directly to the GTP-bound form of Ras [Moodie et al., 1993; Van Aelst et al., 1993; Vojtek et al., 1993; Warne et al., 1993; Zhang et al., 1993], many experiments have established the Raf/MEK/ERK kinase cascade [Crews and Erikson, 1993] as a key growth-stimulating pathway. Experimental evidence suggests that recruitment of Raf kinase to the membrane is the main function of Ras binding [Leevers et al., 1994; Stokoe et al., 1994].

Effector pathways downstream of Ras appear to be numerous and complementary.

Phosphoinositol 3′-kinase (PI3K) represents another effector for Ras [Rodriguez-Viciana et al., 1994]. The observation that products of PI3K promote cellular survival [Yao and Cooper, 1995] suggests that dysregulation of this pathway may also be an important determinant of malignancy. Further investigations suggest that lipid products of PI3K inhibit apoptosis by activating a kinase cascade that includes Atk (also known as protein kinase B [PKB]) [Franke et al., 1995] and its upstream

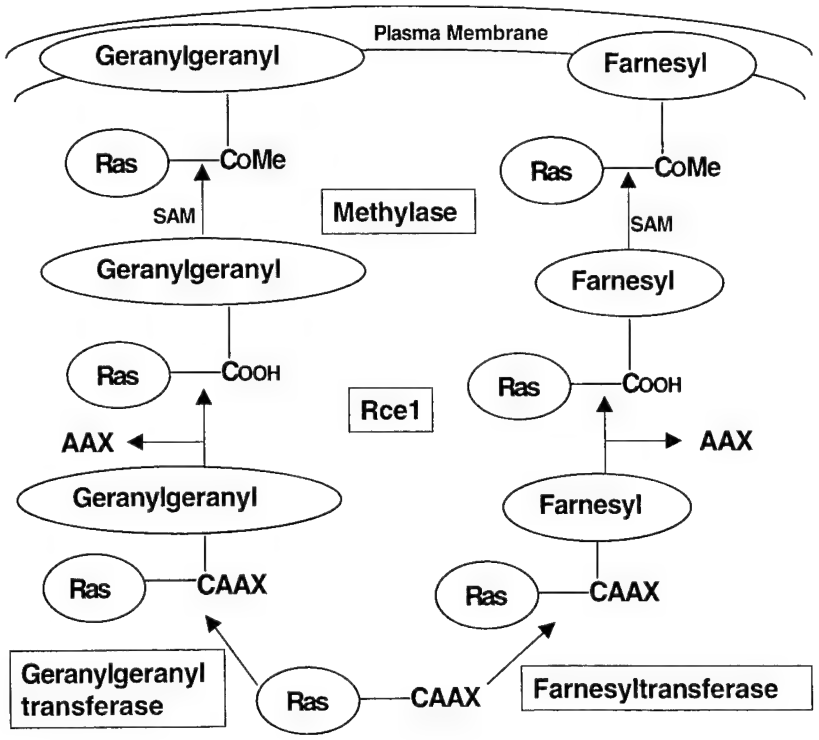

Figure 2. Post-translational processing of Ras. Post-translational modification of Ras is initiated by attachment of either a farnesyl or geranylgeranyl isoprenoid lipid to the cysteine residue of the carboxy terminal CAAX box. These reactions are catalyzed by farnesyl- and geranylgeranyltransferase, respectively. The last three amino acids (i.e., the -A-A-X) are then removed by a specific endoprotease, Rce1. The final step in Ras processing involves methylation of the carboxyl group by an endoplasmic reticulum–associated methyltransferase.

activating kinase phosphoinosotide dependent kinase (PDK) [Alessi et al., 1997; Stokoe et al., 1997].

Roles for additional effectors of Ras have been inferred from studies using Ras effector domain mutants [White et al., 1995; Rodriguez-Viciana et al., 1997]. A set of mutants with differential affinities for a variety of effector proteins suggests that binding to a single effector is insufficient for complete transformation of cells. These studies implicate an effector besides Raf kinase and PI3K. Among the candidates for these additional effectors are Ral GDS, a GEF for the small GTPase Ral [reviewed in Feig et al., 1996]. In addition, there is a growing list of candidates containing a Ral GDS-like Ras binding domain that may also serve as effectors [reviewed in Ponting and Benjamin, 1996]. In spite of the tremendous resources that have been expended to elucidate the Ras signal transduction pathway, it appears likely that additional targets for therapeutic intervention are yet to be discovered.

POST-TRANSLATIONAL PROCESSING OF RAS

Ras proteins undergo post-translational processing at a common C-terminal CAAX sequence where C is cysteine, A is an aliphatic amino acid, and X is any amino acid [Gibbs et al., 1994; Kohl et al., 1995a; Cox and Der, 1997; Gibbs and Oliff, 1997] (Fig. 2). Ras processing is intiated by cytosolic protein prenyltransferases that attach either a farnesyl or a geranylgeranyl isoprenoid lipid to the thiol group of the cysteine residue [Casey and Seabra, 1996]. In general, the cysteine is geranylgeranylated when the X residue is a leucine or a phenylalanine; otherwise, the protein is farnesylated [Finegold et al., 1991; Moores et al., 1991]. The isoprenoid groups that are transferred in these reactions are donated by geranylgeranyl pyrophosphate and by farnesyl pyrophosphate, respectively. This initial lipid modification targets Ras to membranes and is required for the biological activity of both normal and oncogenic Ras. After the prenyl group is attached, the last three amino acids of Ras proteins (i.e., the -A-A-X) are removed by a specific endoprotease [Schmidt et al., 1998]. Recent data from a line of knockout mice suggest that a single gene called *Rce1* encodes the -A-A-X endoprotease activity in mammalian cells (Fig. 2). *Rce1−/−* embryos die late in gestation, and about 50% of the Ras is mislocalized in *Rce1− /−* cells [Kim et al., 1999]. The final step in Ras processing involves methylation of the carboxyl group of the prenylcysteine by an endoplasmic reticulum–associated methyltransferase [Clarke et al., 1988; Clarke, 1992, 1993]. While prenylation of Ras is essential for plasma membrane targeting, biological function, and transformation, the importance of the proteolysis and methylation steps is less certain.

ANTI-RAS THERAPEUTICS

Inhibitors of Ras Processing

A number of farnesyltransferase inhibitors (FTIs) are presently being administered to patients with refractory cancer in Phase 1 and 2 clinical trials. Studies evaluating different FTIs in a number of in vitro and in vivo systems have provided preclinical data supporting selective antitumor effects of these compounds [Cox and Der, 1997; Gibbs and Oliff, 1997]. FTIs have been shown to block Ras-induced transformation in tissue culture cells, to inhibit the growth of many cancer cell lines, and to halt proliferation of Ras-activated xenografts in nude mice [Cox and Der, 1997; Gibbs and Oliff, 1997]. One FTI, L-744,832, also showed efficacy in two transgenic mouse models of breast cancer in which *RAS* expression is driven from a mammary tumor virus (MMTV) promoter [Kohl et al., 1995b; Mangues, 1998]. Barrington and co-

workers [1998] recently reported that L-744,832-induced breast tumor regression was associated with apoptotic cell death that was partially independent of *p53* function in MMTV-*HRAS* mice.

The idea that hyperactive Ras underlies the abnormal cellular proliferation in NF1 provides a rationale for the potential therapeutic value of FTIs and other inhibitors of Ras processing in treating NF1-associated complications. Indeed, Yan and co-workers [1996] found that FTI treatment induced growth inhibition and morphological reversion in an *NF1*-deficient MPNST cell line. We recently investigated the effects of FTI L-744,832 in *Nf1*-deficient hematopoietic cells in vitro and in vivo. L-744,832 inhibited H-Ras prenylation in cell lines and in primary hematopoietic cells and abrogated the growth of myeloid progenitor colonies in response to GM-CSF [Mahgoub et al., 1999]. This FTI also partially blocked GM-CSF-induced MAP kinase activation but did not reduce constitutively elevated levels of MAP kinase activity in primary *Nf1*−/− cells. Injection of a single dose of L-744,832 increased the amount of unprocessed H-Ras in bone marrow cells but had no detectable effect on N-Ras.

Treatment of irradiated recipient mice that had developed the JMML-like myeloid disorder following adoptive transfer of fetal liver cells was not associated with clinical improvement [Mahgoub et al., 1999]. It is likely that the lack of efficacy was due to the resistance of N-Ras and K-Ras processing to inhibition by this FTI. It is not clear if the differences seen when FTIs were tested on MPNST cell lines and in mice engrafted with *Nf1*-deficient hematopoietic cells are accounted for by cell lineage–specific factors, by genetic variations between immortal tumor-derived cell lines and primary cells, by the ability to achieve higher FTI concentrations in tissue culture than in whole animals, or by the use of different compounds in the two studies.

Potential therapeutics targeting Ras effector pathways are not yet in clinical trials. Published inhibitors include two different MEK inhibitors—

Potential therapeutics targeting Ras effector pathways are not yet in clinical trials.

PD98059 [Dudley et al., 1995] and U0126 [Favata et al., 1998]—as well as an inhibitor of PI3K, LY294002 [Vlahos et al., 1994]. The specific effects these inhibitors have on cells are indeed impressive. The MEK inhibitors specifically block Ras-induced ERK phosphorylation and revert many aspects of cellular transformation. The PI3K inhibitor was critical in establishing a role for 3′-phosphorylated lipids in cellular survival pathways; thus, LY294002 is a potent inducer of apoptosis [Yao and Cooper, 1995]. These downstream components of the Ras signaling pathway are thus promising targets for NF1 therapeutics.

Another potential therapeutic strategy for some of the pathogenic complications of NF1 is suggested by data showing that some *NF1*-deficient cells are hypersensitive to specific growth factors. For example, a peptide antagonist of the GM-CSF receptor has been shown to inhibit the growth of human JMML cells both in vitro and in immunodeficient mice [Iversen et al., 1996; Iversen et al., 1997]. As the growth factors that contribute to other hyperproliferative complications of NF1 are elucidated, the development of specific antagonists would represent a rational therapeutic approach for NF1-related tumors.

While gene therapy strategies to correct *NF1* mutations in affected tissues are theoretically appealing, there are significant obstacles to this approach. First, the large size of *NF1* cDNA will make it difficult to construct vectors that can be transduced and expressed efficiently. Using a smaller fragment encoding the *NF1* GAP domain offers a potential solution to this problem; however, it is uncertain if it would be regulated or functional in the same way as full-length neurofibromin. Another problem involves the need to correct a high percentage of cells in multiple lineages. Finally, because Ras plays an integral role in many normal cellular functions, it is likely that exogenous *NF1* will need to be expressed at physiological levels in target cells. In summary, while gene therapy clearly represents an intriguing therapeutic strategy, it is likely that it would be applied to NF1 only after it is validated in such diseases as inherited immunodeficiencies or thalassemia, where the technical issues are less complex. *Nf1* knockout mice may prove useful for modeling specific aspects of gene therapy as applied to NF1.

SUMMARY AND FUTURE PERSPECTIVES

In many ways, NF1 provides an excellent model for assessing strategies that utilize basic information about abnormal cell growth to devise and test rational therapeutics. A large body of genetic and biochemical data implicate hyperactive Ras in many of the pathological complications seen in NF1 patients. Because of its involvement in 30% of human cancers, hyperactive Ras is being intensively investigated as a target for novel drugs. While some of the compounds developed through this effort may prove effective for treating cancers that carry oncogenic *RAS* mutations as well as certain pathological complications of NF1, it is essential that investigators become aware of potential genetic and biochemical differences between these disease states. In particular, oncogenic Ras proteins show defective intrinsic GTP hydrolysis and are resistant to GAPs. In contrast, the Ras proteins present in *NF1*-deficient cells have normal intrinsic biochemical ac-

In many ways, NF1 provides an excellent model for assessing strategies that utilize basic information about abnormal cell growth to devise and test rational therapeutics.

tivities, including responsiveness to p120 GAP. The degree and duration of Ras·GTP activation may differ markedly in cells with different genetic lesions, and this may have profound effects on cellular survival and proliferation [Marshall, 1995; Lloyd et al., 1997; Sewing et al., 1997]. From a therapeutic perspective, it should not be assumed that an agent that is effective or ineffective in cells with oncogenic *RAS* mutations will show efficacy in treating the complications of NF1, and vice versa. If Ras does not function as a simple binary switch in regulating cell growth, reducing Ras·GTP toward (but not all the way back to) normal levels may have unanticipated adverse effects in some cell types.

Despite these potential concerns, anti-Ras therapeutic strategies represent an exciting avenue for NF1 research. The *Nf1* knockout mouse models a number of the important complications of human NF1 and therefore provides a logical system for preclinical studies of novel therapies. These trials should include correlative biochemical studies to ascertain any effects on Ras signaling in primary cells whenever possible. Human clinical trials of new treatments should include a careful assessment of the ratio of risks versus benefits for each patient; this is especially important in NF1, given the great clinical variability between affected individuals. Finally, the development of therapeutic strategies will benefit greatly from continued investments in basic research aimed at defining critical effectors of hyperactive Ras in cell lineages affected in NF1 patients, the role of haplo-insufficiency in various pathological complications, and the contributions of growth factors and of other extracellular stimuli to excessive cellular growth in this disorder.

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RAPID COMMUNICATION

Myeloid Malignancies Induced by Alkylating Agents in *Nf1* Mice

By Nidal Mahgoub, Brigit R. Taylor, Michelle M. Le Beau, Mary Gratiot, Katrin M. Carlson, Susan K. Atwater, Tyler Jacks, and Kevin M. Shannon

Therapy-related acute myeloid leukemia and myelodysplastic syndrome (t-AML and MDS) are severe late complications of treatment with genotoxic chemotherapeutic agents. Children with neurofibromatosis type 1 (NF1) are predisposed to malignant myeloid disorders that are associated with inactivation of the *NF1* tumor suppressor gene in the leukemic clone. Recent clinical data suggest that NF1 might be also associated with an increased risk of t-AML after treatment with alkyating agents. To test this hypothesis, we administered cyclophosphamide or etoposide to cohorts of wild-type and heterozygous *Nf1* knockout mice. Cyclophosphamide exposure cooperated strongly with heterozygous inactivation of *Nf1* in myeloid leukemogenesis, while etoposide did not. Somatic loss of the normal *Nf1* allele correlated with clinical disease and was more common in 129/Sv mice than in 129/Sv × C57BL/6 animals. Leukemic cells showing loss of heterozygosity at *Nf1* retained a structural allele on each chromosome 11 homolog. These studies establish a novel in vivo model of alkylator-induced myeloid malignancy that will facilitate mechanistic and translational studies.

IN 1977, ROWLEY ET AL[1] described a group of patients who developed a myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) as a second malignant neoplasm after treatment for another cancer. Two forms of therapy-related (t) myeloid malignancies are now recognized; these subtypes are associated with distinct clinical and biologic features and differ with respect to the prior anticancer treatment. Most patients with t-AML and t-MDS previously received chemotherapeutic agents that alkylate DNA. This subtype typically involves a latency of 3 to 7 years between genotoxin exposure and disease onset, a myelodysplastic prodrome, and frequent loss of chromosomes 5 and/or 7 (−5 and/or −7) or deletions involving the long arms of these chromosomes [del(5q)/del(7q)].[1-4] The second subtype of t-AML develops after therapy with drugs that inhibit topoisomerase II. These cases are characterized by a shorter interval between cytotoxic therapy and clinical signs, overt leukemia at presentation, and balanced translocations that usually involve the *MLL* gene located on chromosome 11, band q23.[5-8] The prognosis is poor for patients with t-AML or t-MDS. Importantly, as aggressive multi-agent regimens are used increasingly to treat many primary cancers, the incidence of therapy-related myeloid malignancies is expected to increase over the next few years.

Individuals with neurofibromatosis type 1 (NF1) are predisposed to specific benign and malignant neoplasms, which arise primarily in cells derived from the embryonic neural crest.[9] In addition, children (but not adults) with NF1 show a 200 to 500-fold increase in the incidence of de novo malignant myeloid disorders, particularly juvenile myelomonocytic leukemia (JMML).[10-12] The *NF1* gene encodes neurofibromin, a guanosine triphosphatase (GTPase) activating protein that accelerates the slow intrinsic rate of GTP hydrolysis on $p21^{ras}$ (Ras) proteins.[13] Genetic and biochemical data strongly support the hypothesis that *NF1* functions as a tumor suppressor gene in human and murine hematopoietic cells by negatively regulating Ras output.[13-20] For example, approximately 10% of heterozygous *Nf1* knockout mice (*Nf1*+/−) spontaneously develop myeloid leukemia beginning around age 15 months, with tumor cells exhibiting loss of the wild-type *Nf1* allele.[16]

A few cases of t-AML associated with monosomy 7 were recently reported in children with NF1, all of whom received alkylating agents to treat primary cancers including anaplastic astrocytoma, glioblastoma, Wilms' tumor, or acute lymphoblastic leukemia.[21] However, because these patients were not ascertained in a systematic way, it is uncertain if the risk of therapy-related myeloid malignancies is increased over children without NF1 who received similar therapies. Moreover, loss of heterozygosity (LOH) at *NF1* was not detected in the leukemic cells of children with NF1 who developed t-AML.[21] If germline inactivation of *NF1* cooperates with genotoxic agents that are used to treat human cancers in leukemogenesis, we reasoned that exposing *Nf1*+/− mice to these drugs might produce an in vivo model of therapy-induced myeloid disease. To test this hypothesis, we administered two chemotherapeutic agents that have been implicated in human t-AML and t-MDS to *Nf1* mice. Here we show that the alkylating agent cyclophosphamide, but not the toposiomerase II inhibitor etoposide, efficiently induces a myeloproliferative disorder (MPD) in *Nf1*+/− mice, and we present correlative cytogenetic and molecular data.

MATERIALS AND METHODS

Animal care. Mice were housed in the University of California, San Francisco (UCSF) Animal Care facility and were examined regularly by one of the investigators. Cyclophosphamide and etoposide were prepared by the UCSF pharmacy and were administered by one of the

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investigators. Mice were weighed at the beginning of the study and weekly thereafter to adjust the drug doses. Complete blood counts (CBCs) were performed on blood samples collected from tail veins in an automated cell counter. The accuracy of abnormal blood counts was verified by direct examination of stained smears. The study procedures were reviewed and approved by the UCSF Committee for Animal Research.

Treatment and monitoring. We mated *Nf*+/− and wild-type (*Nf*+/+) mice and genotyped the offspring by Southern blot analysis of tail DNA. The initial group of mice were from the inbred 129/Sv strain in which the *Nf1* mutation was created.[16] To perform LOH analysis at loci other than *Nf1* in alkylator-treated mice, F1 offspring of a cross between the 129/Sv and C57BL/6 strains were used in the latter part of the study. *Nf1*+/− and *Nf1*+/+ littermates were assigned to observation (control group) or to receive treatment with either etoposide or cyclophosphamide beginning at 6 to 10 weeks of age. These agents were selected because they are used widely in human cancer therapy. Treated mice received a single 6-week course of 100 mg/kg/wk of either agent, a schedule which approaches the maximally tolerated doses. Cyclophosphamide (CY) was administered by intraperitoneal injection whereas etoposide was administered through an orogastric tube.

CBCs with white blood cell (WBC) differentials were performed every 3 months in mice that appeared well, and whenever a mouse showed signs of systemic illness. The CBC was repeated immediately whenever the WBC count was >20,000/μL. All mice that appeared moribund and animals with WBC counts >20,000/μL on two consecutive determinations were killed, the spleens were weighed, and hematopoietic tissues were collected for morphologic and genetic analysis.

Nf1 genotyping and LOH analysis. Genomic DNA was prepared from tail clippings or from hematopoietic tissues (spleen or bone marrow) by standard procedures.[22] *Nf1* genotypes and loss of heterozygosity were determined by digesting DNA samples with *NcoI* + *Hind*III followed by gel electrophoresis, blotting to nylon membranes, and hybridization with an *NcoI-PstI* fragment from intron 31 of *Nf1* as described previously.[16] LOH was scored by comparing the relative intensities of restriction fragments derived from paired normal and leukemic tissues.

LOH analysis with microsatellite markers. These procedures have been described in detail.[14] Briefly, DNA samples were amplified in a DNA Thermocycle Machine (Perkin Elmer Cetus, Norwalk, CT). Polymerase chain reaction (PCR) was performed in reaction mixtures that include 0.66 μmol/L of respective 3′ and 5′ primers, 100 ng of target genomic DNA, 1 U of Taq polymerase (AmpliTaq; PE Applied Biosystems, Foster City, CA), and 0.4 μmol/L final concentrations of deoxynucleotides in a final reaction volume of 25 μL. The forward primer was kinase-labeled with γ-^{33}P adenosine triphosphate (ATP). Labeled PCR products were separated on (6 mol/L urea, 8% polyacylamide) sequencing-type gels and run at 60 to 80 W constant power for 2 to 4 hours. The gels were dried, placed in Saran wrap (Dow Brands L.P., Indianapolis, IN), and exposed to x-ray film at −70°C. The polymorphic markers tested included D18Mit55, D18Mit13, and D13Mit13, which are syntenic to human 5q31 and D6Mit48, D5Mit40, and D12Mit64, which are syntenic to genes within human 7q22-31.

Cytogenetic analysis and fluorescence in situ hybridization (FISH). A trypsin-Giemsa banding technique was used to analyze cells from bone marrow and spleen. Metaphase cells from short-term (24 to 72 hours) unstimulated cultures were examined. Ten metaphase cells were examined each from the bone marrow and spleen cultures for each mouse. Chromosomes were identified using the standardized mouse karyotype as described by Cowell.[23] FISH was performed as described previously.[24] Briefly, a biotin-labeled *Nf1* probe was prepared by nick-translation using Bio-16-dUTP (Enzo Diagnostics). Hybridization was detected with fluorescein-conjugated avidin (Vector Laboratories), and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole-dihydrochloride (DAPI). Images were obtained using a Zeiss Axiophot microscope coupled to a cooled charge coupled device (CCD) camera. Separate images of DAPI-stained chromosomes and the hybridization signal were merged using image analysis software (NU200, Photometrics Inc, Phoenix, AZ and NIH Image 1.57, National Institutes of Health, Bethesda, MD). The *Nf1* probe used for FISH was a 10.6 kb genomic lambda clone containing exon 31 and flanking intron sequences.

RESULTS

Leukemia in Nf1 +/+ *and* Nf1 +/− *mice.* Myeloid disorders developed in 4 of 101 *Nf1*+/+ mice, 2 of which received CY (Table 1). In contrast, myeloid malignancies were diagnosed in 14% of the untreated *Nf1*+/− mice (8 of 58), in 25% of the etoposide-treated animals (8 of 32), and in 38% (14 of 37) of the mice assigned to the CY group (Table 1). Kaplan-Meier plots comparing disease incidence over time in *Nf1*+/+ and *Nf1*+/− mice that received no treatment, etoposide, or CY are shown in Fig 1. *Nf1*+/− mice that received either drug had a significantly higher rate of disease than wild-type animals treated in parallel (Fig 1). Treated and untreated *Nf1*+/− mice were also compared to ascertain the relative contributions of *Nf1* genotype and chemotherapy exposure to leukemia susceptibility. This analysis showed that the incidence of disease was significantly higher, and the latency period shorter, in the *Nf1*+/− mice that received CY (0.004 *v* untreated *Nf1*+/− mice by pairwise logrank statistics), but not in the etoposide group ($P = .2$ *v* the untreated group). The in vivo leukemogenic effect of CY was restricted to *Nf1*+/− mice as *Nf1*+/+ animals in the control and CY-treated groups had similar rates of leukemia (Table 1). The incidence of leukemia was higher in *Nf1*+/− mice from the inbred 129/Sv background than in 129/Sv × C57BL/6 animals (Table 1), although these differences did not achieve statistical significance.

A myeloproliferative phenotype was observed in most diseased mice that was similar in control and chemotherapy-treated animals. This MPD was characterized by elevated peripheral blood leukocyte counts with a high percentage of mature neutrophils and monocytes (Fig 2). The mean WBC count was 31,000/μL (range, 20,000 to 98,000), and the mean myeloid cell count was 28,000/μL (range, 14,000 to 88,000). Blood smears showed a variable degree of myeloid differentiation with some containing greater than 80% mature neutrophils and others showing 30% to 40% monocytes and monocytoid

Table 1. Incidence of Leukemia in *Nf1* Mice

Genotype and Treatment	Genotype	No. of Mice	No. (%) with Leukemia
129/Sv			
None	*Nf1*+/+	31	2 (6%)
	Nf1+/−	46	8 (17%)
Etoposide	*Nf1*+/+	26	0 (0%)
	Nf1+/−	32	8 (25%)
CY	*Nf1*+/+	5	0 (0%)
	Nf1+/−	12	7 (58%)
129/Sv × C57BL/6			
None	*Nf1*+/+	14	0 (0%)
	Nf1+/−	12	0 (0%)
CY	*Nf1*+/+	25	2 (8%)
	Nf1+/−	25	7 (28%)

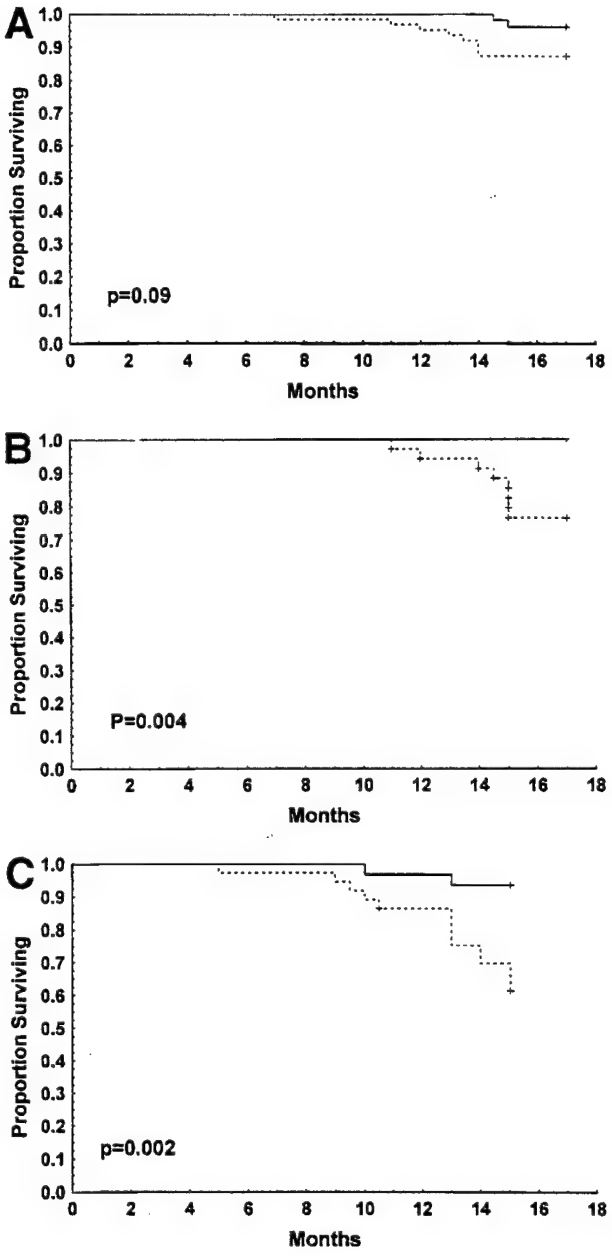

Fig 1. Kaplan-Meier plots showing the proportion of *Nf1*+/+ and *Nf1*+/− mice surviving without leukemia. *Nf1*+/+ mice are shown as an unbroken line and *Nf1*+/− mice as a dotted line. (A) Data from untreated mice; (B) data from the etoposide group; (C) data from the CY group.

cells. Some smears showed rare blasts. Platelet counts and hemoglobin values were normal in mice with MPD and immature erythroid lineage cells were not seen in the peripheral blood. There was no consistent relationship between treatment group, WBC count, and the degree of myeloid maturation visible on blood smears. The bone marrows of mice with MPD showed an overwhelming predominance of myeloid cells with a shift toward immature elements, and sections of the spleen showed expansion of red pulp with infiltration of myeloid cells at various stages of differentiation admixed with areas of erythropoiesis (Fig 2). This MPD is similar to the JMML-like disorder that arises after adoptive transfer of *Nf1*−/− fetal liver cells into irradiated recipient mice.[17] A disease phenotype more consistent with acute leukemia was seen in one CY-treated *Nf1*+/− mouse and in one mouse that received etoposide. Both animals had WBC counts >150,000/μL with large numbers of blasts and few mature neutrophils in the peripheral blood. The CY-treated mouse also had anemia (hemoglobin level, 5.7 g/dL) and thrombocytopenia.

Laboratory investigation of murine leukemias. LOH at *Nf1* correlated with clinical evidence of leukemia in *Nf1*+/− mice (Table 2) and this invariably involved loss of the wild-type *Nf1* allele (Fig 3). Within the CY-treated group, leukemic cells from 129/Sv × C57BL/6 mice showed a much lower incidence of LOH than cells from 129/Sv animals (Table 2). Both animals with evidence of acute leukemia had LOH in hematopoietic tissues. In mice with MPD, LOH was not consistently associated with higher leukocyte counts or with increased numbers of immature myeloid cells. Unexpectedly, we detected LOH at sacrifice in the hematopoietic tissues of 18% of mice that did not fulfill the criteria used to diagnose leukemia. Most of these animals appeared well and WBC counts <10,000/μL and absence of prominent myeloid infiltrates in splenic sections. These results implicate inactivation of *Nf1* as an early event that confers an in vivo proliferative advantage upon a clone of cells, but also suggest that additional mutations are required to produce the characteristic MPD. LOH in the absence of leukemia was much more common in 129/Sv mice than in 129/Sv × C57BL/6 animals (Table 2). Among mice without leukemia, LOH was relatively common in control animals but infrequent in the etoposide-treated cohort (Table 2).

Cytogenetic analysis of bone marrow and spleen cells from 6 mice with MPD (5 CY-treated mice and 1 from the etoposide group) revealed a normal karyotype (Fig 4A). To ascertain if LOH on Southern blots was associated with submicroscopic deletions of *Nf1* or with duplication of the mutant allele, we used a genomic *Nf1* probe from the disrupted segment of the gene to perform FISH analysis of hematopoietic cells from 3 of these 6 mice. FISH showed 2 structural copies of the *Nf1* gene in each case (Fig 4B). We also used six polymorphic microsatellite markers to examine bone marrow DNA from mice with t-ML for LOH at loci syntenic to regions of human chromosomes 5 and 7 that are frequently deleted in humans with t-MDS and t-AML, but found none (data not shown). Similarly, Southern blot analysis of specimens from etoposide-treated mice did not show rearrangements of *Mll* when hybridized with a probe from the human *MLL* breakpoint cluster region that detects virtually all of the breakpoints in human leukemias (data not shown).

DISCUSSION

This study establishes an in vivo model of therapy-induced myeloid malgnancies in *Nf1*+/− mice that will facilitate basic and translational research studies of this important clinical disorder. In human t-MDS/t-AML, frequent deletions involving chromosomes 5 and 7 have implicated loss of gene function in genotoxin-induced leukemogenesis. How alkylating agents actually cause leukemia is unknown; however, CY increases the frequency of somatic inactivation of target genes in a variety of

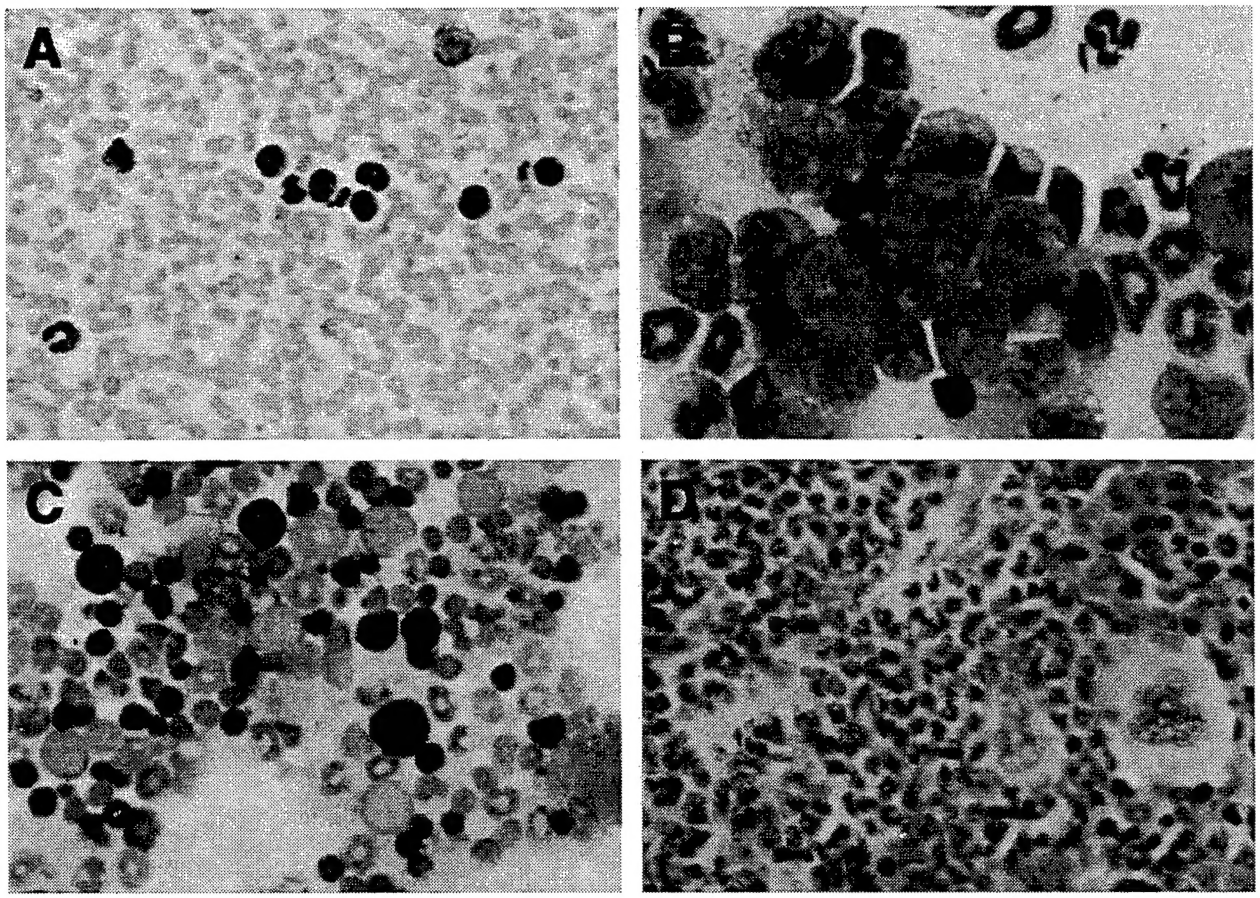

Fig 2. Tissue sections from CY-treated *Nf1*+/− mice with MPD. (A and B) Blood and bone marrow smears showing immature and well differentiated myeloid cells. (C) A cytocentrifuge preparation of spleen cells stained with the myeloid lineage marker nonspecific esterase demonstrates many positive cells (brown stain). (D) A spleen section shows a dense infiltrate of myeloid cells within the red pulp.

assays.[25] In contrast, leukemias that arise after exposure to topoisomerase II inhibitors are associated with recurring chromosomal translocations involving the *MLL* gene that result in the production of dominantly acting chimeric proteins. If the leukemias that develop after treatment with alkylating agents predominately involves the inactivation of specific target genes, it is possible that some human patients who develop t-ML after alkylator-based chemotherapeutic regimens are highly susceptible because of germline mutations of undiscovered tumor-suppressor genes that, like *NF1*, restrain the growth of immature myeloid cells.

We used clinical criteria to diagnose leukemia because the bone marrows of some children with NF1 who develop malignant myeloid disorders do not show LOH at *NF1*.[14,19,21] In this study, mice with clinical evidence of MPD or AML had a threefold higher rate of LOH at *Nf1* than mice without these findings. The presence of somatic LOH in hematopoietic tissues supports the clonal nature of these myeloid disorders. MPDs

Table 2. Loss of Heterozygosity in *Nf1*+/− Mice

Genotype and Treatment	No. of Mice	No. With LOH/ No. With Leukemia	No. With LOH/ No. Without Leukemia
129/Sv			
None	46	5/8 (62%)	10/38 (26%)
Etoposide	32	7/8 (87%)	1/24 (4%)
CY	12	5/7 (71%)	4/5 (80%)
129/Sv × C57BL/6			
None	12	0/0	1/12 (8%)
CY	25	2/7 (28%)	1/18 (5%)
All mice		19/30 (63%)	17/97 (17%)

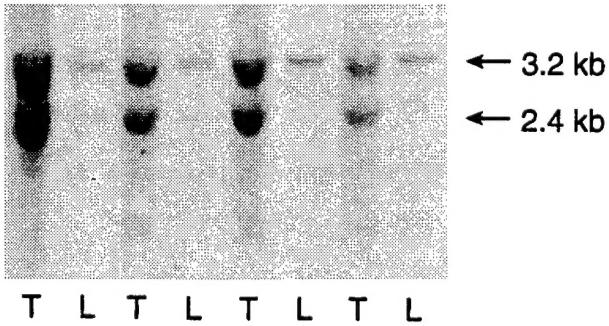

Fig 3. Southern blot analysis of tissues from *Nf1*+/− mice with MPD. The 3.2-kb restriction fragment corresponds to the targeted *Nf1* allele, and the 2.4-kb band is derived from the wild-type allele. DNA extracted from the bone marrows or spleens of five mice with leukemia (L) show absence or a marked reduction in the wild-type *Nf1* allele compared to paired tail (T) DNA specimens.

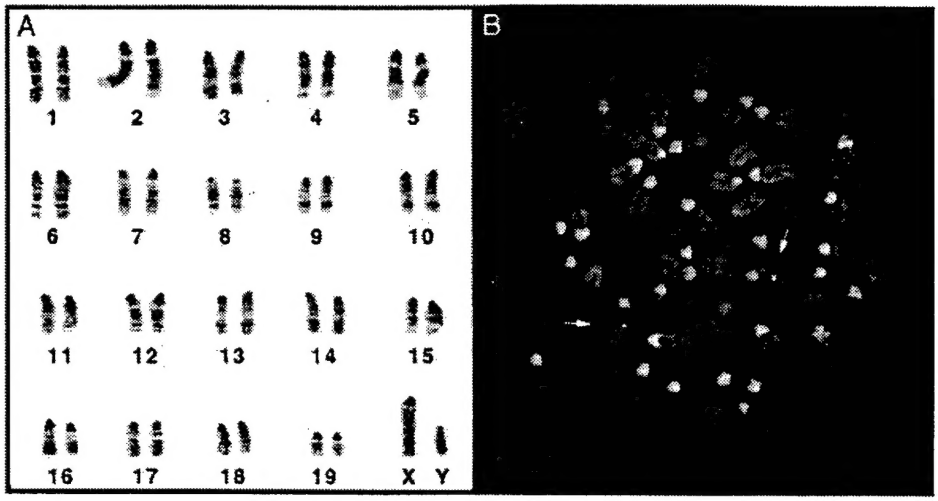

Fig 4. (A) Cytogenetic analysis of spleen cells from a CY-treated mouse with LOH at *Nf1* shows a normal diploid karyotype. (B) FISH of the same specimen shown in (A) with a genomic *Nf1* probe shows 1 copy of the gene on each chromosome 11 homolog.

with and without LOH had similar features to the myeloid disorder that emerges after adoptive transfer of *Nf1*−/− fetal liver cells into irradiated recipient mice.[17] Together with the extraordinary increase in the incidence of leukemia in *Nf1*+/− versus *Nf1*+/+ animals, these observations provide evidence that inactivation of *Nf1* is a central event in leukemogenesis even in the absence of LOH. If this is true, it is likely the wild-type *Nf1* allele is inactivated in bone marrows without LOH by subtle somatic mutations. An alternative consideration is that some *Nf1*+/− mice develop myeloid malignancies through a genetic pathway that does not involve biallelic inactivation of *Nf1*, as has recently been shown for a subset of tumors from heterozygous *p53* knockout mice.[26] Experiments using techniques that can identify point mutations will be required to distinguish between these possibilities. *Nf1* is a very large gene, and protein truncation has proven to be the most efficient method for detecting subtle mutations in normal and leukemic cells from NF1 patients.[20,27,28]

Adoptive transfer of *Nf1*−/− fetal liver cells into irradiated mice consistently induces a MPD with features of JMML.[17] Inasmuch as these data suggested that inactivation of *Nf1* in early hematopoietic cells might be both necessary and sufficient to induce clinical disease, we were surprised to detect LOH at *Nf1* in hematopoietic tissues from 18% of *Nf1*+/− mice with normal WBC counts. This idea that genetic alterations in addition to inactivation of *Nf1* are required for clinical disease is consistent with the relatively long latency between CY exposure and the onset of t-ML in Nf1+/− mice (Fig 1). Cooperating somatic mutations such as bone marrow monosomy 7 and epigenetic events have also been identified in human NF1-associated myeloid disorders.[21,29] It will be of interest to determine if LOH can be detected in circulating blood cells some months before the onset of leukocytosis and splenomegaly in *Nf1*+/− mice.

We did not inject bone marrow cells from *Nf1*+/− mice that acquired myeloid disorders associated with LOH into secondary hosts. In our hands, transferring marrow from recipients previously engrafted with *Nf1*−/− fetal liver cells consistently induces MPD in irradiated, but not in unirradiated, mice (data not shown). In an interesting experiment, Largaespada et al[17] crossed a mutant *Nf1* allele into the BXH2 line of mice in which a leukemogenic retrovirus is transmitted vertically from mother to pups. They observed preferential viral integration into the wild-type *Nf1* allele, shortened latency, and a change in disease phenotype from MPD to AML.[17] Their finding of other somatically acquired leukemia-specific viral integrations within these clones implicated alterations in addition to inactivation of *Nf1* in progression from MPD to AML. Adoptive transfer into secondary recipients provides a way of further characterizing therapy-induced myeloid disorders arising in *Nf1* mice and may be especially informative in rare cases that show features of acute leukemia.

LOH at *Nf1* and clinical leukemia were more common in homozygous 129/Sv mice than in 129/Sv × C57BL/6 animals. This was true in both control and CY-treated mice. Thus, 129/Sv hematopoietic cells are unexpectedly prone to spontaneously undergo LOH at *Nf1* followed by clonal expansion. Rates of cancer in the F1 progeny of crosses between two inbred mouse strains often correlate poorly with parental rates and may be higher, lower, or unchanged.[30] The net effect of our having assigned disproportionate numbers of 129/Sv × C57BL/6 mice to the CY group is to understate the magnitude of the leukemogenic effect of this agent. CY-treated mice showed a higher incidence of clinical leukemia than the control group irrespective of genotype (58% *v* 17% in strain 129/Sv and 28% *v* 0% in strain 129/Sv × C57BL/6; Table 1).

LOH was less frequent in CY-treated 129/Sv × C57BL/6 mice with MPD than in any of the 129/Sv cohorts. This low incidence suggests that the mechanism of *Nf1* inactivation in 129/Sv × C57BL/6 hematopoietic cells involves subtle alkylator-induced mutations rather than loss of the wild-type allele. Consistent with this, Shoemaker et al[31] recently identified somatic *Apc* point mutations caused by transitions or transversions in intestinal tumors from multiple intestinal neoplasia (Min) mice that had been exposed to the alkylating agent N-ethyl-N-nitrosourea (ENU). Interestingly, other tumors from this ENU-exposed cohort showed LOH at *Apc*. Taken together with our data from CY-treated mice, these data suggest that mechanisms of alkylator-induced tumor suppressor gene inactivation in colonic and hematopoietic cells include somatic rearrangements that result in LOH as well as subtle intragenic events.

Intestinal tumors that spontaneously arise in Min mice show LOH at *Apc* with apparent loss of an entire chromosome 18 homolog.[32] However, in a line of mice that carried mutations of the *Apc* and *Dpc4* tumor suppressor genes in *cis,* intestinal tumorigenesis was associated with apparent loss of one entire chromosome homolog with duplication of the mutant chromosome.[33] Consistent with this, FISH analysis of murine leukemias with LOH showed an *Nf1* allele on each chromosome 11 homolog. Although deletion of the chromosome containing the normal tumor suppressor gene allele followed by duplication of the mutant homolog has been proposed as a likely underlying mechanism,[33] other models are also plausible. Mitotic nondisjunction resulting in two copies of the mutant homolog might occur first, with subsequent loss of the normal chromosome. Alternatively, the DNA segment that contains the normal allele might be replaced by a homologous segment from the mutant chromosome by a double mitotic recombination event, as has been reported in a human NF1-associated leukemia.[34]

Haran-Ghera et al[35] previously observed a weak leukemogenic effect of multiple doses of CY when this agent was administered with radiation and dexamethasone to SJL/J mice, a strain that is susceptible to radiation-induced AML. However, CY did not induce leukemia in the absence of radiation, and only cooperated with radiation when it was combined with dexamethasone.[35] In contrast, we have developed a murine model of t-ML based on clinical observations in NF1 patients[21] in which CY alone efficiently induces myeloid leukemia in *Nf1*+/− mice.

Our data provide direct experimental evidence that exposure to a commonly used cancer chemotherapeutic agent can cooperate with a genetic predisposition in the development of myeloid malignancies. Although human patients with t-MDS/t-AML frequently show peripheral blood cytopenias when they seek medical attention, their bone marrows are hypercellular and the disease typically evolves into a frankly proliferative phase with time. Similarly, *Nf1*+/− mice only exhibit overproliferation of myeloid cells months after exposure to CY. As in humans, LOH in murine hematopoietic cells is associated with a copy of the mutant *Nf1* allele on each chromosomal homolog. The relevance of this model to human leukemia is further suggested by the presence of genetic alterations that deregulate Ras signaling in many human myeloid leukemias,[36,37] and by the finding of activating *RAS* mutations in the bone marrows of some patients with t-AML.[38,39] Although our data implicating mutations of genes in addition to *Nf1* in murine leukemogenesis are also consistent with observations in human patients, we did not detect LOH with polymorphic markers from regions of the murine genome that are syntenic to human 5q31 and 7q22. There are a number of potential explanations for these findings, including: (1) the probes used might be some distance from the critical murine loci, (2) the relevant murine genes may be inactivated by somatic mutations which do not result in LOH, (3) loss of DNA sequences synteic to human 5q31 and 7q22 could be late events in progression of MPD to AML that had not occurred by the time of sacrifice, and/or (4) a different spectrum of cooperating genes might be mutated in human and murine leukemias. The nature of the alterations that are involved in alkylator-related leukemias awaits identification of additional target genes in both species.

This novel model provides a rigorous in vivo system to address a number of important (and in some cases controversial) issues in therapy-related myeloid disorders including the relative leukemogenic potential of different alkylating agents, the role of dose intensity, and the additive effects (if any) of alkylating agents and external beam radiotherapy. Furthermore, molecular analysis at *Nf1* may elucidate the mechanistic basis of genetic damage induced by specific alkylating agents in immature hematopoietic cells. *Nf1*+/− knockout mice will also be useful for testing the utility of surrogate markers of gene mutation such as inactivation of *Hprt* to ascertain if exposure to specific mutagens portends an elevated risk of leukemia and to investigate chemopreventive strategies. Finally, these results have implications for the care of individuals with NF1 who develop neoplasms, because they suggest that alkylator-based regimens should be avoided whenever possible.

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